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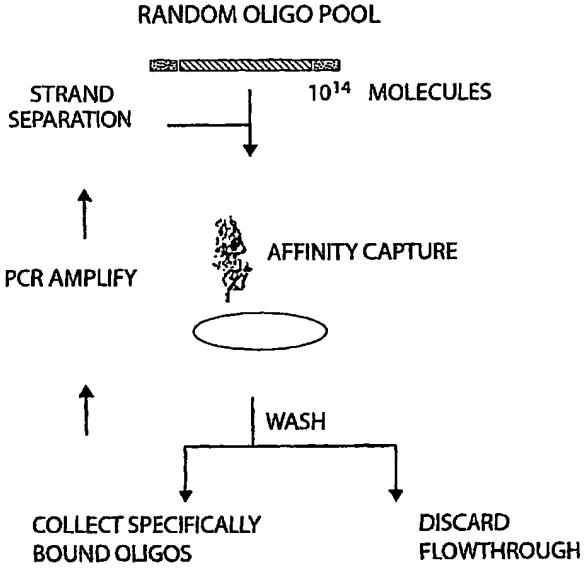
(54) Title: MULTIVALENT APTAMERS



(57) Abstract: Materials and methods are provided for producing aptamer therapeutics having multivalent binding characteristics. The aptamers produced by the methods of the invention are useful in multiple-drug therapeutic applications.

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MULTIVALENT APTAMERS

FIELD OF THE INVENTION

[0001] The invention relates generally to the field of nucleic acids and more particularly to aptamers having multivalent binding characteristics. The invention further relates to materials and methods for making multivalent aptamers to multiple specific targets.

BACKGROUND OF THE INVENTION

[0002] Aptamers are nucleic acid molecules having specific binding affinity to molecules through interactions other than classic Watson-Crick base pairing.

[0003] Aptamers, like peptides generated by phage display or monoclonal antibodies ("MAbs"), are capable of specifically binding to selected targets and modulating the target's activity, *e.g.*, through binding aptamers may block their target's ability to function. Created by an *in vitro* selection process from pools of random sequence oligonucleotides, aptamers have been generated for over 100 proteins including growth factors, transcription factors, enzymes, immunoglobulins, and receptors. A typical aptamer is 10-15 kDa in size (30-45 nucleotides), binds its target with sub-nanomolar affinity, and discriminates against closely related targets (*e.g.*, aptamers will typically not bind other proteins from the same gene family). A series of structural studies have shown that aptamers are capable of using the same types of binding interactions (*e.g.*, hydrogen bonding, electrostatic complementarity, hydrophobic contacts, steric exclusion) that drive affinity and specificity in antibody-antigen complexes.

[0004] Aptamers have a number of desirable characteristics for use as therapeutics and diagnostics including high specificity and affinity, biological efficacy, and excellent pharmacokinetic properties. In addition, they offer specific competitive advantages over antibodies and other protein biologics, for example:

[0005] 1) Speed and control. Aptamers are produced by an entirely *in vitro* process, allowing for the rapid generation of initial leads, including therapeutic leads. *In vitro* selection allows the specificity and affinity of the aptamer to be tightly controlled and allows the generation of leads, including leads against both toxic and non-immunogenic targets.

[0006] 2) Toxicity and Immunogenicity. Aptamers as a class have demonstrated little or no toxicity or immunogenicity. In chronic dosing of rats or woodchucks with high levels of aptamer (10 mg/kg daily for 90 days), no toxicity is observed by any clinical, cellular, or biochemical measure. Whereas the efficacy of many monoclonal antibodies can be severely limited by immune response to antibodies themselves, it is extremely difficult to elicit antibodies to aptamers most likely because aptamers cannot be presented by T-cells via the MHC and the immune response is generally trained not to recognize nucleic acid fragments.

[0007] 3) Administration. Whereas almost all currently approved antibody therapeutics are administered by intravenous infusion (typically over 2-4 hours), aptamers can be administered by subcutaneous injection (aptamer bioavailability via subcutaneous administration is >80% in monkey studies (Tucker *et al.*, J. Chromatography B. 732: 203-212, 1999)). This difference is primarily due to the comparatively low solubility and thus large volumes necessary for most therapeutic MAbs. With good solubility (>150 mg/ml) and comparatively low molecular weight (aptamer: 10-50 kDa; antibody: 150 kDa), a weekly dose of aptamer may be delivered by injection in a volume of less than 0.5 ml. In addition, the small size of aptamers allows them to penetrate into areas of conformational constrictions that do not allow for antibodies or antibody fragments to penetrate, presenting yet another advantage of aptamer-based therapeutics or prophylaxis.

[0008] 4) Scalability and cost. Therapeutic aptamers are chemically synthesized and consequently can be readily scaled as needed to meet production demand. Whereas difficulties in scaling production are currently limiting the availability of some biologics and the capital cost of a large-scale protein production plant is enormous, a single large-scale oligonucleotide synthesizer can produce upwards of 100 kg/year and requires a relatively modest initial investment. The current cost of goods for aptamer synthesis at the kilogram scale is estimated at $500/g, comparable to that for highly optimized antibodies. Continuing improvements in process development are expected to lower the cost of goods to < $100/g in five years.

5) Stability. Therapeutic aptamers are chemically robust. They are intrinsically adapted to regain activity following exposure to factors such as heat and denaturants and can be stored for extended periods (>1 yr) at room temperature as lyophilized powders. In contrast, antibodies must be stored refrigerated.

[0009] There are many disease indications for which multiple targets are implicated. And many current treatment regimens are combinations of therapeutic agents which act synergistically by inhibiting more than one target. Examples include combination therapies for HIV-AIDS and combinations of chemotherapeutic agents and anti-neovascularization agents for cancer indications.

[0010] However, gaining regulatory approval for combination therapies is inherently problematic. In addition to proving that a particular combination is efficacious, it is necessary to prove that the combination of therapeutic agents is superior to the administration of the constituent agents alone. Additionally, the burden of toxicological testing is increased when approval of combination therapies is sought, because the individual components need to be tested separately.

[0011] Given these problems and the advantages of aptamers as therapeutic agents, it would be beneficial to have materials and methods to improve the target valency, pharmacokinetic and pharmacodynamic properties of aptamer therapeutics; and to generate and produce aptamers having multiple binding characteristics useful in addition to or in the place of multiple-drug therapies. The present invention provides materials and methods to meet these and other needs.

BRIEF DESCRIPTION OF THE DRAWINGS

[0012] Figure 1 shows the *in vitro* aptamer selection (SELEX™) process from pools of random sequence oligonucleotides.

[0013] Figure 2(A) shows a schematic of the sequence and secondary structure of a fully 2'-O methylated aptamer (ARC245) capable of binding to VEGF; Figure 2(B) shows a schematic of the sequence and secondary structure of an aptamer (ARC126) capable of binding to PDGF, the semi-circles represent PEG; Figure 2(C) shows a schematic of the sequence and secondary structure of a bivalent aptamer formed by combining the aptamers illustrated in Figures 2(A) and 2(B) capable of binding to VEGF and PDGF in which the PEG linker was substituted with a dGdAdAdA oligonucleotide linker.

[0014] Figure 3(A) shows a schematic of the sequence and secondary structure of a second multivalent aptamer formed by combining the aptamers illustrated in Figures 2(A) and 2(B); Figure 3(B) is a plot showing the proportion of a multivalent aptamer binding to PDGF BB;

Figure 3(C) is a plot showing the proportion of multivalent aptamer binding to VEGF proteins at different protein concentrations.

[0015] Figure 4(A) shows a schematic of the sequence and secondary structure an aptamer capable of binding to TGFβ1 (SEQ ID NO:5); Figure 4(B) shows the sequence and secondary structure of an aptamer capable of binding to TGFβ2 (SEQ ID NO:6); Figure 4(C) shows a schematic of a TGFβ1/TGFβ2 multivalent aptamer (SEQ ID NO:7) constructed from the TGFβ1 and TGFβ2 aptamers shown in Figures 4A and 4B.

[0016] Figure 5(A) is a plot showing the proportion of multivalent aptamer binding to TGFβ1 with a K_D of 14 nM at different protein concentrations and Figure 5(B) is a plot showing the proportion of multivalent aptamer binding to TGFβ2 with a K_D of 400 pM at different protein concentrations.

[0017] Figure 6 illustrates various strategies for synthesis of high molecular weight PEG-nucleic acid conjugates.

[0018] Figure 7 is a chromatographic trace of the synthesis of a 3'-5'-diPEGylated nucleic acid.

[0019] Figure 8A illustrates PDGF bound to a bidentate aptamer ligand stabilized with an oligonucleotide splint; Figure 8B is a binding plot showing the proportion of bound bidentate aptamer to PDGF-BB with respective controls.

[0020] Figure 9A is a binding plot showing the effect of 10 nM splint DNA on the affinity of a PDGF-BB bidentate aptamer; Figure 9B is a binding plot showing the effect of 100 nM splint DNA on the binding affinity of a PDGF-BB bidentate aptamer.

[0021] Figure 10 illustrates the design of a TGFβ2 bidentate aptamer with various spacer compositions and lengths.

[0022] Figure 11 is a binding plot showing the effect of various linker lengths and compositions on the binding of a TGFβ2 bidentate aptamer.

SUMMARY OF THE INVENTION

[0023] The present invention provides materials and methods to generate single oligonucleotide aptamers capable of binding to multiple targets. The present invention also provides single oligonucleotide aptamers capable of binding to multiple targets, *e.g.* capable of binding to more than one of the same target molecule.

[0024] In one embodiment, the present invention provides methods and strategies to design and generate single oligonucleotide aptamers that bind to multiple distinct targets. The present invention also provides single oligonucleotide aptamers capable of binding to multiple distinct targets. In one embodiment, the present invention provides single oligonucleotide aptamers that bind to multiple distinct regions on the same target involved in a disease or disorder.

[0025] In one embodiment, the present invention provides aptamers that bind to multiple different targets involved in a disease or disorder and are useful in multiple-drug therapeutic applications against different targets involved in one disease state.

[0026] In one embodiment, the present invention provides a single oligonucleotide aptamer that has a chemotherapeutic and anti-neovascularization effect and can be useful as a cancer therapeutic.

[0027] In one embodiment, the present invention provides a single oligonucleotide aptamer that binds specifically to vascular endothelial growth factor (VEGF) and platelet derived growth factor (PDGF). In one embodiment, this oligonucleotide is useful as a cancer therapeutic agent.

[0028] In one embodiment, the present invention provides a single oligonucleotide aptamer that binds specifically to TGFβ1 and TGFβ2. In one embodiment, this oligonucleotide is useful as a cancer therapeutic.

[0029] In one embodiment, the present invention provides a single oligonucleotide aptamer that binds specifically to TGFβ1and TGFβ2 and/or PDGF. In one embodiment, this oligonucleotide is useful as an anti-scarring therapeutic.

[0030] In one embodiment, the present invention provides high molecular weight PEG-derivatized nucleic acid (*e.g.*, aptamer) conjugates with improved pharmacological and pharmacodynamic properties and methods for producing such conjugates.

[0031] In one embodiment, the present invention provides high molecular weight PEG-nucleic acid (*e.g.*, aptamer) conjugates and methods for producing such conjugates using a homo-

bifunctional PEG to form a high molecular weight dimer (*i.e.*, a nucleic acid – PEG – nucleic acid conjugate).

[0032] In one embodiment, the present invention provides high molecular weight PEG-nucleic acid (*e.g.*, aptamer) conjugates and methods for producing such conjugates using a bi-reactive nucleic acid (*i.e.*, a nucleic acid bearing two reactive sites) with a mono-functional PEG to form a multiple PEGylated conjugate (*i.e.*, a PEG – nucleic acid – PEG conjugate).

[0033] In one embodiment, the present invention provides oligonucleotide-splinted stabilized multivalent aptamers with enhanced ligand binding properties and methods for producing such conjugates.

[0034] In one embodiment, the present invention provides oligonucleotide-linked multivalent aptamers having improved ligand binding properties and methods for producing such conjugates.

[0035] In one embodiment, the materials and methods of the present invention can be used to generate aptamer molecule multimers that have specificity to a target.

[0036] In one embodiment, the aptamers of the present invention can be used as therapeutics in the prevention and/or treatment of diseases and disorders.

[0037] In one aspect, high molecular weight aptamer compositions of the invention include a nucleic acid having two or more aptamers, and a stabilizing moiety that is a linking moiety, wherein the linking moiety is not a nucleic acid molecule. In one embodiment, the linking moiety is polyalkylene glycol. Suitable polyalkylene glycols include, for example, polyethylene glycol (PEG). In some embodiments, the polyethylene glycol (PEG) linking moiety is multi-activated. For example, the PEG linking moiety is bi-activated. In one embodiment, high molecular weight aptamer compositions include a nucleic acid that has first and second aptamers. In this embodiment, the first and second aptamers are linked by a PEG linking moiety, such that the primary structure of the aptamer composition is a linear arrangement in which the first aptamer is linked to a first terminus of the PEG linking moiety and the second aptamer is linked to a second terminus of the PEG linking moiety. In some embodiments, the high molecular weight aptamer composition has a molecular weight selected from the group consisting of greater than 10 kD, greater than 20 kD, greater than 40 kD and greater than 80 kD.

Some high molecular weight aptamer compositions according to this aspect of the invention are capable of binding to platelet derived growth factor (PDGF). Some high molecular weight aptamer compositions according to this aspect of the invention are capable of binding to TGFβ2.

[0038] In another aspect of the invention, the high molecular weight aptamer compositions include a nucleic acid moiety having two or more aptamer domains joined by a linker domain, and a stabilizing moiety in which one or more polyalkylene glycol moieties attached to the linker domain. In one embodiment, the stabilization moiety is one or more polyalkylene glycol moieties attached to the linker domain. Suitable polyalkylene glycols include, for example, polyethylene glycol (PEG). In some embodiments, the high molecular weight aptamer composition has a molecular weight selected from the group consisting of greater than 10 kD, greater than 20 kD, greater than 40 kD and greater than 80 kD. Some high molecular weight aptamer compositions according to this aspect of the invention are capable of binding to platelet derived growth factor (PDGF). Some high molecular weight aptamer compositions according to this aspect of the invention are capable of binding to TGFβ2.

[0039] In another aspect, the invention provides high molecular weight aptamer compositions that include a nucleic acid having two or more aptamer domains and a linker domain, and a stabilizing moiety that includes an oligonucleotide splint which hybridizes to at least a portion of the linker domain, wherein the oligonucleotide splint has a nucleotide sequence having at least 40 nucleotides. In one embodiment, the oligonucleotide splint hybridizes to at least 20 nucleotides of the linker domain. In some embodiments, the high molecular weight aptamer composition has a molecular weight selected from the group consisting of greater than 10 kD, greater than 20 kD, greater than 40 kD and greater than 80 kD. Some high molecular weight aptamer compositions according to this aspect of the invention are capable of binding to platelet derived growth factor (PDGF). Some high molecular weight aptamer compositions according to this aspect of the invention are capable of binding to TGFβ2.

[0040] In another aspect of the invention, the high molecular weight aptamer compositions include a nucleic acid moiety having two or more aptamer domains and a linker domain, and a stabilizing moiety that includes an oligonucleotide splint that hybridizes to at least a portion of

the linker domain, wherein the oligonucleotide splint has one or more polyalkylene glycol moieties attached thereto. Suitable polyalkylene glycols include, for example, polyethylene glycol (PEG). In one embodiment, the oligonucleotide splint hybridizes to at least 20 nucleotides of the linker domain. In one embodiment, the oligonucleotide splint has a nucleotide sequence having at least 40 nucleotides. In some embodiments, the high molecular weight aptamer composition has a molecular weight selected from the group consisting of greater than 10 kD, greater than 20 kD, greater than 40 kD and greater than 80 kD. Some high molecular weight aptamer compositions according to this aspect of the invention are capable of binding to platelet derived growth factor (PDGF). Some high molecular weight aptamer compositions according to this aspect of the invention are capable of binding to TGFβ2.

[0041] In another aspect, the invention provides high molecular weight aptamer compositions that include a nucleic acid moiety having two or more aptamer domains and a linker domain, and a stabilizing moiety that includes an oligonucleotide splint which hybridizes to at least a portion of the linker domain, wherein at least one of the two or more aptamer domains is in the unbound state (*i.e.*, not bound to a specific aptamer target). In one embodiment, the oligonucleotide splint hybridizes to at least 20 nucleotides of the linker domain. In one embodiment, the oligonucleotide splint has a nucleotide sequence having at least 40 nucleotides. In one embodiment, the oligonucleotide splint has one or more polyalkylene glycol moieties attached thereto. Suitable polyalkylene glycols include, for example, polyethylene glycol (PEG). In some embodiments, the high molecular weight aptamer composition has a molecular weight selected from the group consisting of greater than 10 kD, greater than 20 kD, greater than 40 kD and greater than 80 kD. Some high molecular weight aptamer compositions according to this aspect of the invention are capable of binding to platelet derived growth factor (PDGF). Some high molecular weight aptamer compositions according to this aspect of the invention are capable of binding to TGFβ2.

[0042] In another aspect, the invention provides high molecular weight aptamer compositions that include an aptamer, and two or more non-nucleic acid stabilizing moieties. Suitable stabilizing moieties include, for example, a polyalkylene glycol. In one embodiment, the stabilizing moiety is polyethylene glycol (PEG). In one embodiment, the aptamer is multi-activated. For example, the aptamer is bi-activated.

[0043] The present invention also provides therapeutic compositions. Therapeutic compositions according to the invention include the high molecular weight aptamer compositions described herein.

[0044] In another aspect, the present invention provides methods of improving the pharmacokinetic or pharmacodynamic properties of an aptamer therapeutic composition including the steps of introducing reactive groups in a nucleic acid aptamer, and reacting the reactive groups on the aptamer with reactive groups on a stabilizing moiety, thereby forming a stabilized high molecular weight therapeutic aptamer. In one embodiment, the reactive groups on the aptamer composition are amino groups at 5' or 3' ends of the aptamer introduced by modified phosphoramidite synthesis. In one embodiment, the stabilizing moiety is polyethylene glycol (PEG). In a further embodiment, the PEG is homo-bifunctional and the resulting aptamer is a dimer linked by a PEG linker. In one embodiment, the aptamer is multi-activated. For example, the aptamer is bi-activated. In one embodiment, the aptamer is bi-activated at the 5' and 3' termini. In one embodiment, the stabilizing moiety is a mono-activated PEG and the resulting aptamer is bi-PEGylated.

[0045] In another aspect, the present invention provides methods of treating disease in a subject comprising the steps of administering a therapeutically effective amount of a high molecular weight aptamer compositions described herein.

DETAILED DESCRIPTION OF THE INVENTION

[0046] The details of one or more embodiments of the invention are set forth in the accompanying description below. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials are now described. Other features, objects, and advantages of the invention will be apparent from the description. In the specification, the singular forms also include the plural unless the context clearly dictates otherwise. Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. In the case of conflict, the present Specification will control.

The SELEX™ Method

[0047] A suitable method for generating an aptamer is with the process entitled "Systematic Evolution of Ligands by Exponential Enrichment" ("SELEX™") generally depicted in Figure 1. The SELEX™ process is a method for the *in vitro* evolution of nucleic acid molecules with highly specific binding to target molecules and is described in, *e.g.*, U.S. patent application Ser. No. 07/536,428, filed Jun. 11, 1990, now abandoned, U.S. Pat. No. 5,475,096 entitled "Nucleic Acid Ligands", and U.S. Pat. No. 5,270,163 (see also WO 91/19813) entitled "Nucleic Acid Ligands". Each SELEX™-identified nucleic acid ligand, *i.e.*, each aptamer, is a specific ligand of a given target compound or molecule. The SELEX™ process is based on the unique insight that nucleic acids have sufficient capacity for forming a variety of two- and three-dimensional structures and sufficient chemical versatility available within their monomers to act as ligands (*i.e.*, form specific binding pairs) with virtually any chemical compound, whether monomeric or polymeric. Molecules of any size or composition can serve as targets.

[0048] SELEX™ relies as a starting point upon a large library or pool of single stranded oligonucleotides comprising randomized sequences. The oligonucleotides can be modified or unmodified DNA, RNA, or DNA/RNA hybrids. In some examples, the pool comprises 100% random or partially random oligonucleotides. In other examples, the pool comprises random or partially random oligonucleotides containing at least one fixed and/or conserved sequence incorporated within randomized sequence. In other examples, the pool comprises random or partially random oligonucleotides containing at least one fixed and/or conserved sequence at its 5' and/or 3' end which may comprise a sequence shared by all the molecules of the oligonucleotide pool. Fixed sequences are sequences such as hybridization sites for PCR primers, promoter sequences for RNA polymerases (*e.g.*, T3, T4, T7, and SP6), restriction sites, or homopolymeric sequences, such as poly A or poly T tracts, catalytic cores, sites for selective binding to affinity columns, and other sequences to facilitate cloning and/or sequencing of an oligonucleotide of interest. Conserved sequences are sequences, other than the previously described fixed sequences, shared by a number of aptamers that bind to the same target.

[0049] The oligonucleotides of the pool preferably include a randomized sequence portion as well as fixed sequences necessary for efficient amplification. Typically the oligonucleotides of

the starting pool contain fixed 5' and 3' terminal sequences which flank an internal region of 30–50 random nucleotides. The randomized nucleotides can be produced in a number of ways including chemical synthesis and size selection from randomly cleaved cellular nucleic acids. Sequence variation in test nucleic acids can also be introduced or increased by mutagenesis before or during the selection/amplification iterations.

[0050] The random sequence portion of the oligonucleotide can be of any length and can comprise ribonucleotides and/or deoxyribonucleotides and can include modified or non-natural nucleotides or nucleotide analogs. See, *e.g.*, U.S. Patent No. 5,958,691; U.S. Patent No. 5,660,985; U.S. Patent No. 5,958,691; U.S. Patent No. 5,698,687; U.S. Patent No. 5,817,635; U.S. Patent No. 5,672,695, and PCT Publication WO 92/07065. Random oligonucleotides can be synthesized from phosphodiester-linked nucleotides using solid phase oligonucleotide synthesis techniques well known in the art. See, *e.g.*, Froehler *et al.*, Nucl. Acid Res. 14:5399-5467 (1986) and Froehler *et al.*, Tet. Lett. 27:5575-5578 (1986). Random oligonucleotides can also be synthesized using solution phase methods such as triester synthesis methods. See, *e.g.*, Sood *et al.*, Nucl. Acid Res. 4:2557 (1977) and Hirose *et al.*, Tet. Lett., 28:2449 (1978). Typical syntheses carried out on automated DNA synthesis equipment yield 10^{14}-10^{16} individual molecules, a number sufficient for most SELEX™ experiments. Sufficiently large regions of random sequence in the sequence design increases the likelihood that each synthesized molecule is likely to represent a unique sequence.

[0051] The starting library of oligonucleotides may be generated by automated chemical synthesis on a DNA synthesizer. To synthesize randomized sequences, mixtures of all four nucleotides are added at each nucleotide addition step during the synthesis process, allowing for random incorporation of nucleotides. As stated above, in one embodiment, random oligonucleotides comprise entirely random sequences; however, in other embodiments, random oligonucleotides can comprise stretches of nonrandom or partially random sequences. Partially random sequences can be created by adding the four nucleotides in different molar ratios at each addition step.

[0052] The starting library of oligonucleotides may be either RNA or DNA. In those instances where an RNA library is to be used as the starting library it is typically generated by transcribing a DNA library *in vitro* using T7 RNA polymerase or modified T7 RNA

polymerases and purified. The RNA or DNA library is then mixed with the target under conditions favorable for binding and subjected to step-wise iterations of binding, partitioning and amplification, using the same general selection scheme, to achieve virtually any desired criterion of binding affinity and selectivity. More specifically, starting with a mixture containing the starting pool of nucleic acids, the SELEX™ method includes steps of: (a) contacting the mixture with the target under conditions favorable for binding; (b) partitioning unbound nucleic acids from those nucleic acids which have bound specifically to target molecules; (c) dissociating the nucleic acid-target complexes; (d) amplifying the nucleic acids dissociated from the nucleic acid-target complexes to yield a ligand-enriched mixture of nucleic acids; and (e) reiterating the steps of binding, partitioning, dissociating and amplifying through as many cycles as desired to yield highly specific, high affinity nucleic acid ligands to the target molecule. In those instances where RNA aptamers are being selected, the SELEX™ method further comprises the steps of: (i) reverse transcribing the nucleic acids dissociated from the nucleic acid-target complexes before amplification in step (d); and (ii) transcribing the amplified nucleic acids from step (d) before restarting the process.

[0053] Within a nucleic acid mixture containing a large number of possible sequences and structures, there is a wide range of binding affinities for a given target. A nucleic acid mixture comprising, for example, a 20 nucleotide randomized segment can have 4^{20} candidate possibilities. Those which have the higher affinity constants for the target are most likely to bind to the target. After partitioning, dissociation and amplification, a second nucleic acid mixture is generated, enriched for the higher binding affinity candidates. Additional rounds of selection progressively favor the best ligands until the resulting nucleic acid mixture is predominantly composed of only one or a few sequences. These can then be cloned, sequenced and individually tested for binding affinity as pure ligands or aptamers.

[0054] Cycles of selection and amplification are repeated until a desired goal is achieved. In the most general case, selection/amplification is continued until no significant improvement in binding strength is achieved on repetition of the cycle. The method is typically used to sample approximately 10^{14} different nucleic acid species but may be used to sample as many as about 10^{18} different nucleic acid species. Generally, nucleic acid aptamer molecules are selected in a 5

to 20 cycle procedure. In one embodiment, heterogeneity is introduced only in the initial selection stages and does not occur throughout the replicating process.

[0055] In one embodiment of SELEX™, the selection process is so efficient at isolating those nucleic acid ligands that bind most strongly to the selected target, that only one cycle of selection and amplification is required. Such an efficient selection may occur, for example, in a chromatographic-type process wherein the ability of nucleic acids to associate with targets bound on a column operates in such a manner that the column is sufficiently able to allow separation and isolation of the highest affinity nucleic acid ligands.

[0056] In many cases, it is not necessarily desirable to perform the iterative steps of SELEX™ until a single nucleic acid ligand is identified. The target-specific nucleic acid ligand solution may include a family of nucleic acid structures or motifs that have a number of conserved sequences and a number of sequences which can be substituted or added without significantly affecting the affinity of the nucleic acid ligands to the target. By terminating the SELEX™ process prior to completion, it is possible to determine the sequence of a number of members of the nucleic acid ligand solution family.

[0057] A variety of nucleic acid primary, secondary and tertiary structures are known to exist. The structures or motifs that have been shown most commonly to be involved in non-Watson-Crick type interactions are referred to as hairpin loops, symmetric and asymmetric bulges, pseudoknots and myriad combinations of the same. Almost all known cases of such motifs suggest that they can be formed in a nucleic acid sequence of no more than 30 nucleotides. For this reason, it is often preferred that SELEX™ procedures with contiguous randomized segments be initiated with nucleic acid sequences containing a randomized segment of between about 20to about 50 nucleotides, of about 30 to about 40 nucleotides in some embodiments. In one example, the 5'-fixed:random:3'-fixed sequence comprises a random sequence of about 30 to about 50 nucleotides.

[0058] The core SELEX™ method has been modified to achieve a number of specific objectives. For example, U.S. Patent No. 5,707,796 describes the use of SELEX™ in conjunction with gel electrophoresis to select nucleic acid molecules with specific structural characteristics, such as bent DNA. U.S. Patent No. 5,763,177 describes SELEX™ based methods for selecting nucleic acid ligands containing photoreactive groups capable of binding

and/or photocrosslinking to and/or photoinactivating a target molecule. U.S. Patent No. 5,567,588 and U.S. Patent No. 5,861,254 describe SELEX™ based methods which achieve highly efficient partitioning between oligonucleotides having high and low affinity for a target molecule. U.S. Patent No. 5,496,938 describes methods for obtaining improved nucleic acid ligands after the SELEX™ process has been performed. U.S. Patent No. 5,705,337 describes methods for covalently linking a ligand to its target.

[0059] SELEX™ can also be used to obtain nucleic acid ligands that bind to more than one site on the target molecule, and to obtain nucleic acid ligands that include non-nucleic acid species that bind to specific sites on the target. SELEX™ provides means for isolating and identifying nucleic acid ligands which bind to any envisionable target, including large and small biomolecules such as nucleic acid-binding proteins and proteins not known to bind nucleic acids as part of their biological function as well as cofactors and other small molecules. For example, U.S. Patent No. 5,580,737 discloses nucleic acid sequences identified through SELEX™ which are capable of binding with high affinity to caffeine and the closely related analog, theophylline.

[0060] Counter-SELEX™ is a method for improving the specificity of nucleic acid ligands to a target molecule by eliminating nucleic acid ligand sequences with cross-reactivity to one or more non-target molecules. Counter- SELEX™ is comprised of the steps of: (a) preparing a candidate mixture of nucleic acids; (b) contacting the candidate mixture with the target, wherein nucleic acids having an increased affinity to the target relative to the candidate mixture may be partitioned from the remainder of the candidate mixture; (c) partitioning the increased affinity nucleic acids from the remainder of the candidate mixture; (d) contacting the increased affinity nucleic acids with one or more non-target molecules such that nucleic acid ligands with specific affinity for the non-target molecule(s) are removed; and (e) amplifying the nucleic acids with specific affinity only to the target molecule to yield a mixture of nucleic acids enriched for nucleic acid sequences with a relatively higher affinity and specificity for binding to the target molecule.

[0061] One potential problem encountered in the use of nucleic acids as therapeutics and vaccines is that oligonucleotides in their phosphodiester form may be quickly degraded in body fluids by intracellular and extracellular enzymes such as endonucleases and exonucleases before

the desired effect is manifest. The SELEX™ method thus encompasses the identification of high-affinity nucleic acid ligands containing modified nucleotides conferring improved characteristics on the ligand, such as improved *in vivo* stability or improved delivery characteristics. Examples of such modifications include chemical substitutions at the ribose and/or phosphate and/or base positions. SELEX™-identified nucleic acid ligands containing modified nucleotides are described, *e.g.*, in U.S. Patent No. 5,660,985, which describes oligonucleotides containing nucleotide derivatives chemically modified at the 2' position of ribose, 5 position of pyrimidines, and 8 position of purines, U.S. Patent No. 5,756,703 which describes oligonucleotides containing various 2'-modified pyrimidines, and U.S. Patent No. 5,580,737 which describes highly specific nucleic acid ligands containing one or more nucleotides modified with 2'-amino (2'-NH_2), 2'-fluoro (2'-F), and/or 2'-O-methyl (2'-OMe) substituents.

[0062] Modifications of the nucleic acid ligands contemplated in this invention include, but are not limited to, those which provide other chemical groups that incorporate additional charge, polarizability, hydrophobicity, hydrogen bonding, electrostatic interaction, and fluxionality to the nucleic acid ligand bases or to the nucleic acid ligand as a whole. Modifications to generate oligonucleotide populations which are resistant to nucleases can also include one or more substitute internucleotide linkages, altered sugars, altered bases, or combinations thereof. Such modifications include, but are not limited to, 2'-position sugar modifications, 5-position pyrimidine modifications, 8-position purine modifications, modifications at exocyclic amines, substitution of 4-thiouridine, substitution of 5-bromo or 5-iodo-uracil; backbone modifications, phosphorothioate or alkyl phosphate modifications, methylations, and unusual base-pairing combinations such as the isobases isocytidine and isoguanidine. Modifications can also include 3' and 5' modifications such as capping.

[0063] In one embodiment, oligonucleotides are provided in which the P(O)O group is replaced by P(O)S ("thioate"), P(S)S ("dithioate"), P(O)NR_2 ("amidate"), P(O)R, P(O)OR', CO or CH_2 ("formacetal") or 3'-amine (-NH-CH_2-CH_2-), wherein each R or R' is independently H or substituted or unsubstituted alkyl. Linkage groups can be attached to adjacent nucleotides through an -O-, -N-, or -S- linkage. Not all linkages in the oligonucleotide are required to be identical.

[0064] In further embodiments, the oligonucleotides comprise modified sugar groups, for example, one or more of the hydroxyl groups is replaced with halogen, aliphatic groups, or functionalized as ethers or amines. In one embodiment, the 2'-position of the furanose residue is substituted by any of an O-methyl, O-alkyl, O-allyl, S-alkyl, S-allyl, or halo group. Methods of synthesis of 2'-modified sugars are described, *e.g.*, in Sproat, *et al.*, Nucl. Acid Res. 19:733-738 (1991); Cotten, *et al.*, Nucl. Acid Res. 19:2629-2635 (1991); and Hobbs, *et al.*, Biochemistry 12:5138-5145 (1973). Other modifications are known to one of ordinary skill in the art. Such modifications may be pre-SELEX™ process modifications or post-SELEX™ process modifications (modification of previously identified unmodified ligands) or may be made by incorporation into the SELEX process.

[0065] Pre-SELEX process modifications or those made by incorporation into the SELEX process yield nucleic acid ligands with both specificity for their SELEX™ target and improved stability, *e.g.*, *in vivo* stability. Post-SELEX™ process modifications made to nucleic acid ligands may result in improved stability, *e.g.*, *in vivo* stability without adversely affecting the binding capacity of the nucleic acid ligand.

[0066] The SELEX™ method encompasses combining selected oligonucleotides with other selected oligonucleotides and non-oligonucleotide functional units as described in U.S. Patent No. 5,637,459 and U.S. Patent No. 5,683,867. The SELEX™ method further encompasses combining selected nucleic acid ligands with lipophilic or non-immunogenic high molecular weight compounds in a diagnostic or therapeutic complex, as described, *e.g.*, in U.S. Patent No. 6,011,020, U.S. Patent No. 6,051,698, and PCT Publication No. WO 98/18480. These patents and applications teach the combination of a broad array of shapes and other properties, with the efficient amplification and replication properties of oligonucleotides, and with the desirable properties of other molecules.

[0067] The identification of nucleic acid ligands to small, flexible peptides via the SELEX™ method has also been explored. Small peptides have flexible structures and usually exist in solution in an equilibrium of multiple conformers, and thus it was initially thought that binding affinities may be limited by the conformational entropy lost upon binding a flexible peptide. However, the feasibility of identifying nucleic acid ligands to small peptides in solution was

demonstrated in U.S. Patent No. 5,648,214. In this patent, high affinity RNA nucleic acid ligands to substance P, an 11 amino acid peptide, were identified.

[0068] The aptamers with specificity and binding affinity to the target(s) of the present invention are typically selected by the SELEX™ process as described herein. As part of the SELEX™ process, the sequences selected to bind to the target are then optionally minimized to determine the minimal sequence having the desired binding affinity. The selected sequences and/or the minimized sequences are optionally optimized by performing random or directed mutagenesis of the sequence to increase binding affinity or alternatively to determine which positions in the sequence are essential for binding activity. Additionally, selections can be performed with sequences incorporating modified sequences to stabilize the aptamer molecules against degradation *in vivo*.

2'Modified SELEX™

[0069] In order for an aptamer to be suitable for use as a therapeutic, it is preferably inexpensive to synthesize, safe and stable *in vivo*. Wild-type RNA and DNA aptamers are typically not stable *in vivo* because of their susceptibility to degradation by nucleases. Resistance to nuclease degradation can be greatly increased by the incorporation of modifying groups at the 2'-position.

[0070] Fluoro and amino groups have been successfully incorporated into oligonucleotide libraries from which aptamers have been subsequently selected. However, these modifications greatly increase the cost of synthesis of the resultant aptamer, and may introduce safety concerns in some cases because of the possibility that the modified nucleotides could be recycled into host DNA by degradation of the modified oligonucleotides and subsequent use of the nucleotides as substrates for DNA synthesis.

[0071] Aptamers that contain 2'-O-methyl (2'-OMe) nucleotides, as provided herein, overcome many of these drawbacks. Oligonucleotides containing 2'-O-methyl nucleotides are nuclease-resistant and inexpensive to synthesize. Although 2'-O-methyl nucleotides are ubiquitous in biological systems, natural polymerases do not accept 2'-O-methyl NTPs as substrates under physiological conditions, thus there are no safety concerns over the recycling of 2'-O-methyl nucleotides into host DNA. The SELEX™ method used to generate 2'-modified aptamers is

described, *e.g.*, in U.S. Provisional Patent Application Serial No. 60/430,761, filed December 3, 2002, U.S. Provisional Patent Application Serial No. 60/487,474, filed July 15, 2003, U.S. Provisional Patent Application Serial No. 60/517,039, filed November 4, 2003, U.S. Patent Application No. 10/729,581, filed December 3, 2003, and U.S. Patent Application No. 10/873,856, filed June 21, 2004, entitled "Method for *in vitro* Selection of 2'-O-methyl Substituted Nucleic Acids", each of which is herein incorporated by reference in its entirety.

[0072] The present invention includes multivalent aptamers that bind to and modulate the function of at least two of PDGF, VEGF, TGFβ1 or TGFβ2 which contain modified nucleotides (*e.g.*, nucleotides which have a modification at the 2'position) to make the oligonucleotide more stable than the unmodified oligonucleotide to enzymatic and chemical degradation as well as thermal and physical degradation. Although there are several examples of 2'-OMe containing aptamers in the literature (see, *e.g.*, Green et al., Current Biology 2, 683-695, 1995) these were generated by the *in vitro* selection of libraries of modified transcripts in which the C and U residues were 2'-fluoro (2'-F) substituted and the A and G residues were 2'-OH. Once functional sequences were identified then each A and G residue was tested for tolerance to 2'-OMe substitution, and the aptamer was re-synthesized having all A and G residues which tolerated 2'-OMe substitution as 2'-OMe residues. Most of the A and G residues of aptamers generated in this two-step fashion tolerate substitution with 2'-OMe residues, although, on average, approximately 20% do not. Consequently, aptamers generated using this method tend to contain from two to four 2'-OH residues, and stability and cost of synthesis are compromised as a result. By incorporating modified nucleotides into the transcription reaction which generate stabilized oligonucleotides used in oligonucleotide libraries from which aptamers are selected and enriched by SELEX™ (and/or any of its variations and improvements, including those described herein), the methods of the present invention eliminate the need for stabilizing the selected aptamer oligonucleotides *(e.g.*, by resynthesizing the aptamer oligonucleotides with modified nucleotides).

[0073] In one embodiment, the present invention provides aptamers comprising combinations of 2'-OH, 2'-F, 2'-deoxy, and 2'-OMe modifications of the ATP, GTP, CTP, TTP, and UTP nucleotides. In another embodiment, the present invention provides aptamers comprising combinations of 2'-OH, 2'-F, 2'-deoxy, 2'-OMe, 2'-NH_2, and 2'-methoxyethyl modifications of

the ATP, GTP, CTP, TTP, and UTP nucleotides. In another embodiment, the present invention provides aptamers comprising 5^6 combinations of 2'-OH, 2'-F, 2'-deoxy, 2'-OMe, 2'-NH_2, and 2'-methoxyethyl modifications of the ATP, GTP, CTP, TTP, and UTP nucleotides.

[0074] 2' modified aptamers of the invention are created using modified polymerases, *e.g.*, a modified T7 polymerase, having a rate of incorporation of modified nucleotides having bulky substituents at the furanose 2' position that is higher than that of wild-type polymerases. For example, a single mutant T7 polymerase (Y639F) in which the tyrosine residue at position 639 has been changed to phenylalanine readily utilizes 2'deoxy, 2'amino-, and 2'fluoro- nucleotide triphosphates (NTPs) as substrates and has been widely used to synthesize modified RNAs for a variety of applications. However, this mutant T7 polymerase reportedly can not readily utilize (*i.e.*, incorporate) NTPs with bulky 2'-substituents such as 2'-OMe or 2'-azido (2'-N_3) substituents. For incorporation of bulky 2' substituents, a double T7 polymerase mutant (Y639F/H784A) having the histidine at position 784 changed to an alanine residue in addition to the Y639F mutation has been described and has been used in limited circumstances to incorporate modified pyrimidine NTPs. See Padilla, R. and Sousa, R., Nucleic Acids Res., 2002, 30(24): 138. A single mutant T7 polymerase (H784A) having the histidine at position 784 changed to an alanine residue has also been described. Padilla *et al.*, Nucleic Acids Research, 2002, 30: 138. In both the Y639F/H784A double mutant and H784A single mutant T7 polymerases, the change to a smaller amino acid residue such as alanine allows for the incorporation of bulkier nucleotide substrates, *e.g.*, 2'-O methyl substituted nucleotides.

[0075] Generally, it has been found that under the conditions disclosed herein, the Y693F single mutant can be used for the incorporation of all 2'-OMe substituted NTPs except GTP and the Y639F/H784A double mutant can be used for the incorporation of all 2'-OMe substituted NTPs including GTP. It is expected that the H784A single mutant possesses properties similar to the Y639F and the Y639F/H784A mutants when used under the conditions disclosed herein.

[0076] 2'-modified oligonucleotides may be synthesized entirely of modified nucleotides, or with a subset of modified nucleotides. The modifications can be the same or different. All nucleotides may be modified, and all may contain the same modification. All nucleotides may be modified, but contain different modifications, *e.g.*, all nucleotides containing the same base

may have one type of modification, while nucleotides containing other bases may have different types of modification. All purine nucleotides may have one type of modification (or are unmodified), while all pyrimidine nucleotides have another, different type of modification (or are unmodified). In this way, transcripts, or libraries of transcripts are generated using any combination of modifications, including for example, ribonucleotides (2'-OH), deoxyribonucleotides (2'-deoxy), 2'-F, and 2'-OMe nucleotides. A transcription mixture containing 2'-OMe C and U and 2'-OH A and G is referred to as a "rRmY" mixture and aptamers selected therefrom are referred to as "rRmY" aptamers. A transcription mixture containing deoxy A and G and 2'-OMe U and C is referred to as a "dRmY" mixture and aptamers selected therefrom are referred to as "dRmY" aptamers. A transcription mixture containing 2'-OMe A, C, and U, and 2'-OH G is referred to as a "rGmH" mixture and aptamers selected therefrom are referred to as "rGmH" aptamers. A transcription mixture alternately containing 2'-OMe A, C, U and G and 2'-OMe A, U and C and 2'-F G is referred to as a "toggle" mixture and aptamers selected therefrom are referred to as "toggle" aptamers. A transcription mixture containing 2'-OMe A, U, C, and G, where up to 10% of the G's are ribonucleotides is referred to as a "r/mGmH" mixture and aptamers selected therefrom are referred to as "r/mGmH" aptamers. A transcription mixture containing 2'-OMe A, U, and C, and 2'-F G is referred to as a "fGmH" mixture and aptamers selected therefrom are referred to as "fGmH" aptamers. A transcription mixture containing 2'-OMe A, U, and C, and deoxy G is referred to as a "dGmH" mixture and aptamers selected therefrom are referred to as "dGmH" aptamers. A transcription mixture containing deoxy A, and 2'-OMe C, G and U is referred to as a "dAmB" mixture and aptamers selected therefrom are referred to as "dAmB" aptamers, and a transcription mixture containing all 2'-OH nucleotides is referred to as a "rN" mixture and aptamers selected therefrom are referred to as "rN" or "rRrY" aptamers. A "mRmY" aptamer is one containing all 2'-O-methyl nucleotides and is usually derived from a r/mGmH oligonucleotide by post-SELEX replacement, when possible, of any 2'-OH Gs with 2'-OMe Gs.

[0077] A preferred embodiment includes any combination of 2'-OH, 2'-deoxy and 2'-OMe nucleotides. A more preferred embodiment includes any combination of 2'-deoxy and 2'-OMe

nucleotides. An even more preferred embodiment is with any combination of 2'-deoxy and 2'-OMe nucleotides in which the pyrimidines are 2'-OMe (such as dRmY, mRmY or dGmH).

[0078] Incorporation of modified nucleotides into the aptamers of the invention is accomplished before (pre-) the selection process (*e.g.*, a pre-SELEX™ process modification). Optionally, aptamers of the invention in which modified nucleotides have been incorporated by pre-SELEX™ process modification can be further modified by post-SELEX™ process modification (*i.e.*, a post-SELEX™ process modification after a pre-SELEX™ modification). Pre-SELEX™ process modifications yield modified nucleic acid ligands with specificity for the SELEX™ target and also improved *in vivo* stability. Post-SELEX™ process modifications, *i.e.*, modification (*e.g.*, truncation, deletion, substitution or additional nucleotide modifications of previously identified ligands having nucleotides incorporated by pre-SELEX™ process modification) can result in a further improvement of *in vivo* stability without adversely affecting the binding capacity of the nucleic acid ligand having nucleotides incorporated by pre-SELEX™ process modification.

[0079] To generate libraries of 2'-modified (*e.g.*, 2'-OMe) RNA transcripts in conditions under which a polymerase accepts 2'-modified NTPs the preferred polymerase is the Y693F/H784A double mutant or the Y693F single mutant. Other polymerases, particularly those that exhibit a high tolerance for bulky 2'-substituents, may also be used in the present invention. Such polymerases can be screened for this capability by assaying their ability to incorporate modified nucleotides under the transcription conditions disclosed herein.

[0080] A number of factors have been determined to be important for the transcription conditions useful in the methods disclosed herein. For example, increases in the yields of modified transcript are observed when a leader sequence is incorporated into the 5' end of a fixed sequence at the 5' end of the DNA transcription template, such that at least about the first 6 residues of the resultant transcript are all purines.

[0081] Another important factor in obtaining transcripts incorporating modified nucleotides is the presence or concentration of 2'-OH GTP. Transcription can be divided into two phases: the first phase is initiation, during which an NTP is added to the 3'-hydroxyl end of GTP (or another substituted guanosine) to yield a dinucleotide which is then extended by about 10-12 nucleotides; the second phase is elongation, during which transcription proceeds beyond the

addition of the first about 10-12 nucleotides. It has been found that small amounts of 2'-OH GTP added to a transcription mixture containing an excess of 2'-OMe GTP are sufficient to enable the polymerase to initiate transcription using 2'-OH GTP, but once transcription enters the elongation phase the reduced discrimination between 2'-OMe and 2'-OH GTP, and the excess of 2'-OMe GTP over 2'-OH GTP allows the incorporation of principally the 2'-OMe GTP.

[0082] Another important factor in the incorporation of 2'-OMe substituted nucleotides into transcripts is the use of both divalent magnesium and manganese in the transcription mixture. Different combinations of concentrations of magnesium chloride and manganese chloride have been found to affect yields of 2'-O-methylated transcripts, the optimum concentration of the magnesium and manganese chloride being dependent on the concentration in the transcription reaction mixture of NTPs which complex divalent metal ions. To obtain the greatest yields of maximally 2' substituted O-methylated transcripts (*i.e.*, all A, C, and U and about 90% of G nucleotides), concentrations of approximately 5 mM magnesium chloride and 1.5 mM manganese chloride are preferred when each NTP is present at a concentration of 0.5 mM. . When the concentration of each NTP is 1.0 mM, concentrations of approximately 6.5 mM magnesium chloride and 2.0 mM manganese chloride are preferred. When the concentration of each NTP is 2.0 mM, concentrations of approximately 9.6 mM magnesium chloride and 2.9 mM manganese chloride are preferred. In any case, departures from these concentrations of up to two-fold still give significant amounts of modified transcripts.

[0083] Priming transcription with GMP or guanosine is also important. This effect results from the specificity of the polymerase for the initiating nucleotide. As a result, the 5'-terminal nucleotide of any transcript generated in this fashion is likely to be 2'-OH G. The preferred concentration of GMP (or guanosine) is 0.5 mM and even more preferably 1 mM. It has also been found that including PEG, preferably PEG-8000, in the transcription reaction is useful to maximize incorporation of modified nucleotides.

[0084] For maximum incorporation of 2'-OMe ATP (100%), UTP (100%), CTP (100%) and GTP (~90%) ("r/mGmH") into transcripts the following conditions are preferred: HEPES buffer 200 mM, DTT 40 mM, spermidine 2 mM, PEG-8000 10% (w/v), Triton X-100 0.01%

(w/v), $MgCl_2$ 5 mM (6.5 mM where the concentration of each 2'-OMe NTP is 1.0 mM), $MnCl_2$ 1.5 mM (2.0 mM where the concentration of each 2'-OMe NTP is 1.0 mM), 2'-OMe NTP (each) 500 μM (more preferably, 1.0 mM), 2'-OH GTP 30 μM, 2'-OH GMP 500 μM, pH 7.5, Y639F/H784A T7 RNA Polymerase 15 units/ml, inorganic pyrophosphatase 5 units/ml, and an all-purine leader sequence of at least 8 nucleotides long. As used herein, one unit of the Y639F/H784A mutant T7 RNA polymerase (or any other mutant T7 RNA polymerase specified herein) is defined as the amount of enzyme required to incorporate 1 nmole of 2'-OMe NTPs into transcripts under the r/mGmH conditions. As used herein, one unit of inorganic pyrophosphatase is defined as the amount of enzyme that will liberate 1.0 mole of inorganic orthophosphate per minute at pH 7.2 and 25 °C.

[0085] For maximum incorporation (100%) of 2'-OMe ATP, UTP and CTP ("rGmH") into transcripts the following conditions are preferred: HEPES buffer 200 mM, DTT 40 mM, spermidine 2 mM, PEG-8000 10% (w/v), Triton X-100 0.01% (w/v), $MgCl_2$ 5 mM (9.6 mM where the concentration of each 2'-OMe NTP is 2.0 mM), $MnCl_2$ 1.5 mM (2.9 mM where the concentration of each 2'-OMe NTP is 2.0 mM), 2'-OMe NTP (each) 500 μM (more preferably, 2.0 mM), pH 7.5, Y639F T7 RNA Polymerase 15 units/ml, inorganic pyrophosphatase 5 units/ml, and an all-purine leader sequence of at least 8 nucleotides long.

[0086] For maximum incorporation (100%) of 2'-OMe UTP and CTP ("rRmY") into transcripts the following conditions are preferred: HEPES buffer 200 mM, DTT 40 mM, spermidine 2 mM, PEG-8000 10% (w/v), Triton X-100 0.01% (w/v), $MgCl_2$ 5 mM (9.6 mM where the concentration of each 2'-OMe NTP is 2.0 mM), $MnCl_2$ 1.5 mM (2.9 mM where the concentration of each 2'-OMe NTP is 2.0 mM), 2'-OMe NTP (each) 500μM (more preferably, 2.0 mM), pH 7.5, Y639F/H784A T7 RNA Polymerase 15 units/ml, inorganic pyrophosphatase 5 units/ml, and an all-purine leader sequence of at least 8 nucleotides long.

[0087] For maximum incorporation (100%) of deoxy ATP and GTP and 2'-OMe UTP and CTP ("dRmY") into transcripts the following conditions are preferred: HEPES buffer 200 mM, DTT 40 mM, spermine 2 mM, spermidine 2 mM, PEG-8000 10% (w/v), Triton X-100 0.01% (w/v), $MgCl_2$ 9.6 mM, $MnCl_2$ 2.9 mM, 2'-OMe NTP (each) 2.0 mM, pH 7.5, Y639F T7 RNA

Polymerase 15 units/ml, inorganic pyrophosphatase 5 units/ml, and an all-purine leader sequence of at least 8 nucleotides long.

[0088] For maximum incorporation (100%) of 2'-OMe ATP, UTP and CTP and 2'-F GTP ("fGmH") into transcripts the following conditions are preferred: HEPES buffer 200 mM, DTT 40 mM, spermidine 2 mM, PEG-8000 10% (w/v), Triton X-100 0.01% (w/v), $MgCl_2$ 9.6 mM, $MnCl_2$ 2.9 mM, 2'-OMe NTP (each) 2.0 mM, pH 7.5, Y639F T7 RNA Polymerase 15 units/ml, inorganic pyrophosphatase 5 units/ml, and an all-purine leader sequence of at least 8 nucleotides long.

[0089] For maximum incorporation (100%) of deoxy ATP and 2'-OMe UTP, GTP and CTP ("dAmB") into transcripts the following conditions are preferred: HEPES buffer 200 mM, DTT 40 mM, spermidine 2 mM, PEG-8000 10% (w/v), Triton X-100 0.01% (w/v), $MgCl_2$ 9.6 mM, $MnCl_2$ 2.9 mM, 2'-OMe NTP (each) 2.0 mM, pH 7.5, Y639F T7 RNA Polymerase 15 units/ml, inorganic pyrophosphatase 5 units/ml, and an all-purine leader sequence of at least 8 nucleotides long.

[0090] For each of the above (a) transcription is preferably performed at a temperature of from about 20 °C to about 50 °C, preferably from about 30 °C to 45 °C, and more preferably at about 37 °C for a period of at least two hours and (b) 50-300 nM of a double stranded DNA transcription template is used (200 nM template is used in round 1 to increase diversity (300 nM template is used in dRmY transcriptions)), and for subsequent rounds approximately 50 nM, a 1/10 dilution of an optimized PCR reaction, using conditions described herein, is used). The preferred DNA transcription templates are described below (where ARC254 and ARC256 transcribe under all 2'-OMe conditions and ARC255 transcribes under rRmY conditions).

[0091] ARC254 (SEQ ID NO:17):

5'-CATCGATGCTAGTCGTAACGATCCNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNCGAGAACGTTCTCTCCTCTCCCTATAGTGAGTCGTATTA-3'

ARC255 (SEQ ID NO:18):

5'-CATGCATCGCGACTGACTAGCCGNNNNNNNNNNNNNNNNNNNNNNNNN
NNNNNGTAGAACGTTCTCTCCTCTCCCTATAGTGAGTCGTATTA-3'

ARC256 (SEQ ID NO:19):

5'-CATCGATCGATCGATCGACAGCGNNNNNNNNNNNNNNNNNNNNNNNNN
NNNNNGTAGAACGTTCTCTCCTCTCCCTATAGTGAGTCGTATTA–3'

[0092] Under rN transcription conditions of the present invention, the transcription reaction mixture comprises 2′-OH adenosine triphosphates (ATP), 2′-OH guanosine triphosphates (GTP), 2′-OH cytidine triphosphates (CTP), and 2′-OH uridine triphosphates (UTP). The modified oligonucleotides produced using the rN transcription mixtures of the present invention comprise substantially all 2′-OH adenosine, 2′-OH guanosine, 2′-OH cytidine, and 2′-OH uridine. In a preferred embodiment of rN transcription, the resulting modified oligonucleotides comprise a sequence where at least 80% of all adenosine nucleotides are 2′-OH adenosine, at least 80% of all guanosine nucleotides are 2′-OH guanosine, at least 80% of all cytidine nucleotides are 2′-OH cytidine, and at least 80% of all uridine nucleotides are 2′-OH uridine. In a more preferred embodiment of rN transcription, the resulting modified oligonucleotides of the present invention comprise a sequence where at least 90% of all adenosine nucleotides are 2′-OH adenosine, at least 90% of all guanosine nucleotides are 2′-OH guanosine, at least 90% of all cytidine nucleotides are 2′-OH cytidine, and at least 90% of all uridine nucleotides are 2′-OH uridine. In a most preferred embodiment of rN transcription, the modified oligonucleotides of the present invention comprise a sequence where 100% of all adenosine nucleotides are 2′-OH adenosine, 100% of all guanosine nucleotides are 2′-OH guanosine, 100% of all cytidine nucleotides are 2′-OH cytidine, and 100% of all uridine nucleotides are 2′-OH uridine.

[0093] Under rRmY transcription conditions of the present invention, the transcription reaction mixture comprises 2′-OH adenosine triphosphates, 2′-OH guanosine triphosphates, 2′-O-methyl cytidine triphosphates, and 2′-O-methyl uridine triphosphates. The modified oligonucleotides produced using the rRmY transcription mixtures of the present invention comprise substantially all 2′-OH adenosine, 2′-OH guanosine, 2′-O-methyl cytidine and 2′-O-methyl uridine. In a preferred embodiment, the resulting modified oligonucleotides comprise a sequence where at

least 80% of all adenosine nucleotides are 2′-OH adenosine, at least 80% of all guanosine nucleotides are 2′-OH guanosine, at least 80% of all cytidine nucleotides are 2′-O-methyl cytidine and at least 80% of all uridine nucleotides are 2′-O-methyl uridine. In a more preferred embodiment, the resulting modified oligonucleotides comprise a sequence where at least 90% of all adenosine nucleotides are 2′-OH adenosine, at least 90% of all guanosine nucleotides are 2′-OH guanosine, at least 90% of all cytidine nucleotides are 2′-O-methyl cytidine and at least 90% of all uridine nucleotides are 2′-O-methyl uridine In a most preferred embodiment, the resulting modified oligonucleotides comprise a sequence where 100% of all adenosine nucleotides are 2′-OH adenosine, 100% of all guanosine nucleotides are 2′-OH guanosine, 100% of all cytidine nucleotides are 2′-O-methyl cytidine and 100% of all uridine nucleotides are 2′-O-methyl uridine.

[0094] Under dRmY transcription conditions of the present invention, the transcription reaction mixture comprises 2′-deoxy purine triphosphates and 2′-O-methyl pyrimidine triphosphates. The modified oligonucleotides produced using the dRmY transcription conditions of the present invention comprise substantially all 2'-deoxy purines and 2′-O-methyl pyrimidines. In a preferred embodiment, the resulting modified oligonucleotides of the present invention comprise a sequence where at least 80% of all purine nucleotides are 2'-deoxy purines and at least 80% of all pyrimidine nucleotides are 2′-O-methyl pyrimidines. In a more preferred embodiment, the resulting modified oligonucleotides of the present invention comprise a sequence where at least 90% of all purine nucleotides are 2'-deoxy purines and at least 90% of all pyrimidine nucleotides are 2′-O-methyl pyrimidines. In a most preferred embodiment, the resulting modified oligonucleotides of the present invention comprise a sequence where 100% of all purine nucleotides are 2'-deoxy purines and 100% of all pyrimidine nucleotides are 2′-O-methyl pyrimidines.

[0095] Under rGmH transcription conditions of the present invention, the transcription reaction mixture comprises 2′-OH guanosine triphosphates, 2′-O-methyl cytidine triphosphates, 2′-O-methyl uridine triphosphates, and 2′-O-methyl adenosine triphosphates. The modified oligonucleotides produced using the rGmH transcription mixtures of the present invention comprise substantially all 2′-OH guanosine, 2′-O-methyl cytidine, 2′-O-methyl uridine, and 2′-O-methyl adenosine. In a preferred embodiment, the resulting modified oligonucleotides

comprise a sequence where at least 80% of all guanosine nucleotides are 2′-OH guanosine, at least 80% of all cytidine nucleotides are 2′-O-methyl cytidine, at least 80% of all uridine nucleotides are 2′-O-methyl uridine, and at least 80% of all adenosine nucleotides are 2′-O-methyl adenosine. In a more preferred embodiment, the resulting modified oligonucleotides comprise a sequence where at least 90% of all guanosine nucleotides are 2′-OH guanosine, at least 90% of all cytidine nucleotides are 2′-O-methyl cytidine, at least 90% of all uridine nucleotides are 2′-O-methyl uridine, and at least 90% of all adenosine nucleotides are 2′-O-methyl adenosine. In a most preferred embodiment, the resulting modified oligonucleotides comprise a sequence where 100% of all guanosine nucleotides are 2′-OH guanosine, 100% of all cytidine nucleotides are 2′-O-methyl cytidine, 100% of all uridine nucleotides are 2′-O-methyl uridine, and 100% of all adenosine nucleotides are 2′-O-methyl adenosine.

[0096] Under r/mGmH transcription conditions of the present invention, the transcription reaction mixture comprises 2′-O-methyl adenosine triphosphate, 2′-O-methyl cytidine triphosphate, 2′-O-methyl guanosine triphosphate, 2′-O-methyl uridine triphosphate and 2'-OH guanosine triphosphate. The resulting modified oligonucleotides produced using the r/mGmH transcription mixtures of the present invention comprise substantially all 2′-O-methyl adenosine, 2′-O-methyl cytidine, 2′-O-methyl guanosine, and 2′-O-methyl uridine, wherein the population of guanosine nucleotides has a maximum of about 10% 2'-OH guanosine. In a preferred embodiment, the resulting r/mGmH modified oligonucleotides of the present invention comprise a sequence where at least 80% of all adenosine nucleotides are 2′-O-methyl adenosine, at least 80% of all cytidine nucleotides are 2′-O-methyl cytidine, at least 80% of all guanosine nucleotides are 2′-O-methyl guanosine, at least 80% of all uridine nucleotides are 2′-O-methyl uridine, and no more than about 10% of all guanosine nucleotides are 2'-OH guanosine. In a more preferred embodiment, the resulting modified oligonucleotides comprise a sequence where at least 90% of all adenosine nucleotides are 2′-O-methyl adenosine, at least 90% of all cytidine nucleotides are 2′-O-methyl cytidine, at least 90% of all guanosine nucleotides are 2′-O-methyl guanosine, at least 90% of all uridine nucleotides are 2′-O-methyl uridine, and no more than about 10% of all guanosine nucleotides are 2'-OH guanosine. In a most preferred embodiment, the resulting modified oligonucleotides comprise a sequence

where 100% of all adenosine nucleotides are 2′-O-methyl adenosine, 100% of all cytidine nucleotides are 2′-O-methyl cytidine, 90% of all guanosine nucleotides are 2′-O-methyl guanosine, and 100% of all uridine nucleotides are 2′-O-methyl uridine, and no more than about 10% of all guanosine nucleotides are 2'-OH guanosine.

[0097] Under fGmH transcription conditions of the present invention, the transcription reaction mixture comprises 2′-O-methyl adenosine triphosphates, 2′-O-methyl uridine triphosphates, 2′-O-methyl cytidine triphosphates, and 2′-F guanosine triphosphates. The modified oligonucleotides produced using the fGmH transcription conditions of the present invention comprise substantially all 2′-O-methyl adenosine, 2′-O-methyl uridine, 2′-O-methyl cytidine, and 2′-F guanosine. In a preferred embodiment, the resulting modified oligonucleotides comprise a sequence where at least 80% of all adenosine nucleotides are 2′-O-methyl adenosine, at least 80% of all uridine nucleotides are 2′-O-methyl uridine, at least 80% of all cytidine nucleotides are 2′-O-methyl cytidine, and at least 80% of all guanosine nucleotides are 2′-F guanosine. In a more preferred embodiment, the resulting modified oligonucleotides comprise a sequence where at least 90% of all adenosine nucleotides are 2′-O-methyl adenosine, at least 90% of all uridine nucleotides are 2′-O-methyl uridine, at least 90% of all cytidine nucleotides are 2′-O-methyl cytidine, and at least 90% of all guanosine nucleotides are 2′-F guanosine. In a most preferred embodiment, the resulting modified oligonucleotides comprise a sequence where 100% of all adenosine nucleotides are 2′-O-methyl adenosine, 100% of all uridine nucleotides are 2′-O-methyl uridine, 100% of all cytidine nucleotides are 2′-O-methyl cytidine, and 100% of all guanosine nucleotides are 2′-F guanosine.

[0098] Under dAmB transcription conditions of the present invention, the transcription reaction mixture comprises 2′-deoxy adenosine triphosphates, 2′-O-methyl cytidine triphosphates (CTP), 2′-O-methyl guanosine triphosphates, and 2′-O-methyl uridine triphosphates. The modified oligonucleotides produced using the dAmB transcription mixtures of the present invention comprise substantially all 2′-deoxy adenosine, 2′-O-methyl cytidine, 2′-O-methyl guanosine, and 2′-O-methyl uridine. In a preferred embodiment, the resulting modified oligonucleotides comprise a sequence where at least 80% of all adenosine nucleotides are 2′-deoxy adenosine, at least 80% of all cytidine nucleotides are 2′-O-methyl cytidine, at least 80%

of all guanosine nucleotides are 2′-O-methyl guanosine, and at least 80% of all uridine nucleotides are 2′-O-methyl uridine. In a more preferred embodiment, the resulting modified oligonucleotides comprise a sequence where at least 90% of all adenosine nucleotides are 2′-deoxy adenosine, at least 90% of all cytidine nucleotides are 2′-O-methyl cytidine, at least 90% of all guanosine nucleotides are 2′-O-methyl guanosine, and at least 90% of all uridine nucleotides are 2′-O-methyl uridine. In a most preferred embodiment, the resulting modified oligonucleotides of the present invention comprise a sequence where 100% of all adenosine nucleotides are 2′-deoxy adenosine, 100% of all cytidine nucleotides are 2′-O-methyl cytidine, 100% of all guanosine nucleotides are 2′-O-methyl guanosine, and 100% of all uridine nucleotides are 2′-O-methyl uridine.

[0099] In each case, the transcription products can then be used as the library in the SELEX™ process to identify aptamers and/or to determine a conserved motif of sequences that have binding specificity to a given target. The resulting sequences are already stabilized, eliminating this step from the process to arrive at a stabilized aptamer sequence and giving a more highly stabilized aptamer as a result. Another advantage of the 2'-OMe SELEX™ process is that the resulting sequences are likely to have fewer 2'-OH nucleotides required in the sequence, possibly none. To the extent 2'OH nucleotides remain they can be removed by performing post-SELEX modifications.

[00100] As described below, lower but still useful yields of transcripts fully incorporating 2' substituted nucleotides can be obtained under conditions other than the optimized conditions described above. For example, variations to the above transcription conditions include:

[00101] The HEPES buffer concentration can range from 0 to 1 M. The present invention also contemplates the use of other buffering agents having a pKa between 5 and 10 including, for example, Tris(hydroxymethyl)aminomethane.

[00102] The DTT concentration can range from 0 to 400 mM. The methods of the present invention also provide for the use of other reducing agents including, for example, mercaptoethanol.

[00103] The spermidine and/or spermine concentration can range from 0 to 20 mM.

[00104] The PEG-8000 concentration can range from 0 to 50 % (w/v). The methods of the present invention also provide for the use of other hydrophilic polymer including, for example, other molecular weight PEG or other polyalkylene glycols.

[00105] The Triton X-100 concentration can range from 0 to 0.1% (w/v). The methods of the present invention also provide for the use of other non-ionic detergents including, for example, other detergents, including other Triton-X detergents.

[00106] The $MgCl_2$ concentration can range from 0.5 mM to 50 mM. The $MnCl_2$ concentration can range from 0.15 mM to 15 mM. Both $MgCl_2$ and $MnCl_2$ must be present within the ranges described and in a preferred embodiment are present in about a 10 to about 3 ratio of $MgCl_2$:$MnCl_2$, preferably, the ratio is about 3-5:1, more preferably, the ratio is about 3-4:1.

[00107] The 2'-OMe NTP concentration (each NTP) can range from 5 μM to 5 mM.

[00108] The 2'-OH GTP concentration can range from 0 μM to 300 μM.

[00109] The 2'-OH GMP concentration can range from 0 to 5 mM.

[00110] The pH can range from pH 6 to pH 9. The methods of the present invention can be practiced within the pH range of activity of most polymerases that incorporate modified nucleotides. In addition, the methods of the present invention provide for the optional use of chelating agents in the transcription reaction condition including, for example, EDTA, EGTA, and DTT.

[00111] The selected aptamers having the highest affinity and specific binding as demonstrated by biological assays as described in the examples below are suitable therapeutics for treating conditions in which the target is involved in pathogenesis.

Multivalent Aptamers

[00112] As used herein the term multivalent aptamers (*e.g.* bivalent, trivalent, etc.) refers to molecules comprising at least two aptamers or at least two aptamer domains that are capable of binding to at least two different targets, at least two distinct regions on the same target, or at least two of the same target, *e.g.*, bind to the same regions on the same targets. In some embodiments, at least two aptamer domains are a part of a single multivalent aptamer. In particular embodiments the single multivalent aptamer does not comprise a linker. While in

other embodiments, at least two aptamers are chemically linked *e.g.*, by PEG, a single stranded oligonucleotide linker, or a splinted oligonucleotide linker, to form a multivalent aptamer molecule comprising at least two chemically linked aptamers.

[00113] As used herein, the term bidentate aptamers encompasses multivalent aptamers comprising two distinct domains capable of binding to the same target, and multivalent aptamers comprising two of the same domains capable of binding to the same target.

[00114] As used herein, multimeric aptamers refers to a multivalent aptamer comprising at least two of the same domain capable of binding to the same target.

[00115] The materials of the present invention comprise a series of multivalent nucleic acid aptamer of 34-166 nucleotides in length which bind specifically to multiple targets *e.g.*, at least two of PDGF, VEGF, TGFβ1, or TGFβ2 and which functionally modulate, *e.g.*, block, the activity of PDGF, VEGF, TGFβ1, and/or TGFβ2 in *in vivo* and/or cell-based assays.

[00116] Aptamers specifically capable of binding and modulating at least two of PDGF, VEGF, TGFβ1, or TGFβ2 are set forth herein. These aptamers provide a low-toxicity, safe, and effective modality of treating and/or preventing diseases or disorders such as cancer, angiogenesis, and scarring, which are known to be caused by or otherwise associated with PDGF, VEGF, TGFβ1, and/or TGFβ2.

[00117] Examples of multivalent aptamers for use as therapeutics and/or diagnostics include the following sequences: SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:7, SEQ ID NO:9, and SEQ ID NO:10.

[00118] The multivalent aptamers that bind at least two of PDGF, VEGF, TGFβ1, or TGFβ2 are described below in Examples 1-6.

[00119] These multivalent aptamers may include modifications as described herein including, *e.g.*, conjugation to lipophilic or high molecular weight compounds (*e.g.*, PEG, incorporation of a CpG motif, incorporation of a capping moiety, and incorporation of modified nucleotides.

[00120] In one embodiment of the invention an isolated, non-naturally occurring bidentate aptamer that binds to PDGF is provided. In another embodiment, the bidentate aptamer of the invention modulates a function of PDGF. In another embodiment, the bidentate aptamer of the invention inhibits a target function of PDGF while in another embodiment the bidentate aptamer stimulates a function of PDGF. In another embodiment of the invention, the bidentate

aptamer binds and/or modulates a function of a PDGF variant. A PDGF variant as used herein encompasses variants that perform essentially the same function as PDGF, preferably comprises substantially the same structure and in some embodiments comprises 80% sequence identity, more preferably 90% sequence identity, and more preferably 95% sequence identity to the amino acid sequence of PDGF.

[00121] In another embodiment of the invention, the bidentate aptamer has substantially the same ability to bind PDGF as that of an aptamer comprising SEQ ID NO:9. In another embodiment of the invention, the bidentate aptamer has substantially the same structure and ability to bind PDGF as that of an aptamer comprising SEQ ID NO:9. In another embodiment, the bidentate aptamer of the invention has a sequence according to SEQ ID NO:9. In another embodiment, the bidentate aptamers of the invention are used as an active ingredient in pharmaceutical compositions. In another embodiment, the bidentate aptamers or compositions comprising the bidentate aptamers of the invention are used to treat cancer and angiogenesis.

[00122] In one embodiment of the invention an isolated, non-naturally occurring bidentate aptamer that binds to TGFβ2 is provided. In another embodiment, the bidentate aptamer of the invention modulates a function of TGFβ2. In another embodiment, the bidentate aptamer of the invention inhibits a function of TGFβ2 while in another embodiment the bidentate aptamer stimulates a function of the TGFβ2. In another embodiment of the invention, the bidentate aptamer binds and/or modulates a function of a TGFβ2 variant. A TGFβ2 variant as used herein encompasses variants that perform essentially the same function as TGFβ2, preferably comprises substantially the same structure and in some embodiments comprises 80% sequence identity, more preferably 90% sequence identity, and more preferably 95% sequence identity to the amino acid sequence of TGFβ2.

[00123] In another embodiment of the invention, the bidentate aptamer has substantially the same ability to bind TGFβ2 as that of an aptamer comprising SEQ ID NO:10. In another embodiment of the invention, the bidentate aptamer has substantially the same structure and ability to bind TGFβ2 as that of an aptamer comprising SEQ ID NO:10. In another embodiment, the bidentate aptamer of the invention has a sequence according to SEQ ID NO:10. In another embodiment, the bidentate aptamers of the invention are used as an active

ingredient in pharmaceutical compositions. In another embodiment, the bidentate aptamers or compositions comprising the aptamers of the invention are used to treat scarring.

[00124] In one embodiment of the invention an isolated, non-naturally occurring multivalent aptamer that binds to PDGF and VEGF is provided. In another embodiment, the multivalent aptamer of the invention modulates a function of PDGF and/or VEGF. In another embodiment, the multivalent aptamer of the invention inhibits a target function while in another embodiment the multivalent aptamer stimulates a function of the target. In another embodiment of the invention, the multivalent aptamer binds and/or modulates a function of a PDGF and/or a VEGF variant. A PDGF or VEGF variant as used herein encompasses variants that perform essentially the same function as PDGF or VEGF, preferably comprises substantially the same structure and in some embodiments comprises 80% sequence identity, more preferably 90% sequence identity, and more preferably 95% sequence identity to the amino acid sequences of PDGF and VEGF.

[00125] In another embodiment of the invention, the multivalent aptamer has substantially the same ability to bind PDGF and VEGF as that of an aptamer comprising any one of SEQ ID NOS: 3 and 4. In another embodiment of the invention, the multivalent aptamer has substantially the same structure and ability to bind PDGF and VEGF as that of an aptamer comprising any one of SEQ ID NOS: 3 and 4. In another embodiment, the multivalent aptamers of the invention have a sequence according to any one of SEQ ID NOS:3 and 4. In another embodiment, the multivalent aptamers of the invention are used as an active ingredient in pharmaceutical compositions. In another embodiment, the multivalent aptamers or compositions comprising the aptamers of the invention are used to treat cancer and angiogenesis.

[00126] In one embodiment of the invention an isolated, non-naturally occurring multivalent aptamer that binds to TGFβ1 and TGFβ2 is provided. In another embodiment, the multivalent aptamer of the invention modulates a function of TGFβ1 and/or TGFβ2. In another embodiment, the multivalent aptamer of the invention inhibits a target function while in another embodiment the multivalent aptamer stimulates a function of the target. In another embodiment of the invention, the multivalent aptamer binds and/or modulates a function of a TGFβ1 and/or a TGFβ2 variant. A TGFβ1 or TGFβ2 variant as used herein encompasses

variants that perform essentially the same function as TGFβ1 or TGFβ2, preferably comprises substantially the same structure and in some embodiments comprises 80% sequence identity, more preferably 90% sequence identity, and more preferably 95% sequence identity to the amino acid sequences of TGFβ1 and TGFβ2.

[00127] In another embodiment of the invention, the multivalent aptamer has substantially the same ability to bind TGFβ1 and TGFβ2 as that of an aptamer comprising SEQ ID NO:7. In another embodiment of the invention, the multivalent aptamer has substantially the same structure and ability to bind TGFβ1 and TGFβ2 as that of an aptamer comprising SEQ ID NO:7. In another embodiment, the multivalent aptamers of the invention has a sequence according to SEQ ID NO:7. In another embodiment, the multivalent aptamers of the invention are used as an active ingredient in pharmaceutical compositions. In another embodiment, the multivalent aptamers or compositions comprising the multivalent aptamers of the invention are used to treat scarring.

[00128] In some embodiments aptamer therapeutics of the present invention have great affinity and specificity to their targets while reducing the deleterious side effects from non-naturally occurring nucleotide substitutions if the aptamer therapeutics break down in the body of patients or subjects. In some embodiments, the therapeutic compositions containing the aptamer therapeutics of the present invention are free of or have a reduced amount of fluorinated nucleotides.

[00129] The aptamers of the present invention can be synthesized using any oligonucleotide synthesis techniques known in the art including solid phase oligonucleotide synthesis techniques well known in the art (see, *e.g.*, Froehler *et al.*, Nucl. Acid Res. 14:5399-5467 (1986) and Froehler *et al.*, Tet. Lett. 27:5575-5578 (1986)) and solution phase methods such as triester synthesis methods (see, *e.g.*, Sood *et al.*, Nucl. Acid Res. 4:2557 (1977) and Hirose *et al.*, Tet. Lett., 28:2449 (1978)).

Pharmaceutical Compositions

[00130] The invention also includes pharmaceutical compositions containing multivalent aptamer molecules that bind to PDGF, VEGF, TGFβ1, or TGFβ2. In some embodiments, the compositions are suitable for internal use and include an effective amount of a

pharmacologically active compound of the invention, alone or in combination, with one or more pharmaceutically acceptable carriers. The compounds are especially useful in that they have very low, if any toxicity.

[00131] Compositions of the invention can be used to treat or prevent a pathology, such as a disease or disorder, or alleviate the symptoms of such disease or disorder in a patient. For example, compositions of the present invention can be used to treat or prevent a pathology associated with cancer, angiogenesis, and scarring. Compositions of the invention are useful for administration to a subject suffering from, or predisposed to, a disease or disorder which is related to or derived from a target to which the aptamers of the invention specifically bind.

[00132] Compositions of the invention can be used in a method for treating a patient or subject having a pathology. The method involves administering to the patient or subject an aptamer or a composition comprising aptamers that bind a target (*e.g.*, a protein) involved with the pathology, so that binding of the aptamer to the target alters the biological function of the target, thereby treating the pathology.

[00133] The patient or subject having a pathology, *i.e.,* the patient or subject treated by the methods of this invention can be a mammal, more particularly a vertebrate, or more particularly, a human.

[00134] In practice, the aptamers or their pharmaceutically acceptable salts, are administered in amounts which will be sufficient to exert their desired biological activity, *e.g.*, inhibiting the binding of the aptamer target to its receptor.

[00135] One aspect of the invention comprises an aptamer composition of the invention in combination with other treatments for PDGF, VEGF, TGFβ1, and TGFβ2 related disorders. The aptamer composition of the invention may contain, for example, more than one aptamer. In some examples, an aptamer composition of the invention, containing one or more compounds of the invention, is administered in combination with another useful composition such as an anti-inflammatory agent, an immunosuppressant, an antiviral agent, or the like. Furthermore, the compounds of the invention may be administered in combination with a cytotoxic, cytostatic, or chemotherapeutic agent such as an alkylating agent, anti-metabolite, mitotic inhibitor or cytotoxic antibiotic, as described above. In general, the currently available dosage forms of the known therapeutic agents for use in such combinations will be suitable.

[00136] "Combination therapy" (or "co-therapy") includes the administration of an aptamer composition of the invention and at least a second agent as part of a specific treatment regimen intended to provide the beneficial effect from the co-action of these therapeutic agents. The beneficial effect of the combination includes, but is not limited to, pharmacokinetic or pharmacodynamic co-action resulting from the combination of therapeutic agents. Administration of these therapeutic agents in combination typically is carried out over a defined time period (usually minutes, hours, days or weeks depending upon the combination selected).

[00137] "Combination therapy" may, but generally is not, intended to encompass the administration of two or more of these therapeutic agents as part of separate monotherapy regimens that incidentally and arbitrarily result in the combinations of the present invention. "Combination therapy" is intended to embrace administration of these therapeutic agents in a sequential manner, that is, wherein each therapeutic agent is administered at a different time, as well as administration of these therapeutic agents, or at least two of the therapeutic agents, in a substantially simultaneous manner. Substantially simultaneous administration can be accomplished, for example, by administering to the subject a single capsule having a fixed ratio of each therapeutic agent or in multiple, single capsules for each of the therapeutic agents.

[00138] Sequential or substantially simultaneous administration of each therapeutic agent can be effected by any appropriate route including, but not limited to, topical routes, oral routes, intravenous routes, intramuscular routes, and direct absorption through mucous membrane tissues. The therapeutic agents can be administered by the same route or by different routes. For example, a first therapeutic agent of the combination selected may be administered by injection while the other therapeutic agents of the combination may be administered topically.

[00139] Alternatively, for example, all therapeutic agents may be administered topically or all therapeutic agents may be administered by injection. The sequence in which the therapeutic agents are administered is not narrowly critical unless noted otherwise. "Combination therapy" also can embrace the administration of the therapeutic agents as described above in further combination with other biologically active ingredients. Where the combination therapy further comprises a non-drug treatment, the non-drug treatment may be conducted at any suitable time so long as a beneficial effect from the co-action of the combination of the therapeutic agents and non-drug treatment is achieved. For example, in appropriate cases, the beneficial effect is

still achieved when the non-drug treatment is temporally removed from the administration of the therapeutic agents, perhaps by days or even weeks.

[00140] Therapeutic or pharmacological compositions of the present invention will generally comprise an effective amount of the active component(s) of the therapy, dissolved or dispersed in a pharmaceutically acceptable medium. Pharmaceutically acceptable media or carriers include any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents and the like. The use of such media and agents for pharmaceutical active substances is well known in the art. Supplementary active ingredients can also be incorporated into the therapeutic compositions of the present invention.

[00141] The preparation of pharmaceutical or pharmacological compositions will be known to those of skill in the art in light of the present disclosure. Typically, such compositions may be prepared as injectables, either as liquid solutions or suspensions; solid forms suitable for solution in, or suspension in, liquid prior to injection; as tablets or other solids for oral administration; as time release capsules; or in any other form currently used, including eye drops, creams, lotions, salves, inhalants and the like. The use of sterile formulations, such as saline-based washes, by surgeons, physicians or health care workers to treat a particular area in the operating field may also be particularly useful. Compositions may also be delivered via microdevice, microparticle or sponge.

[00142] Upon formulation, therapeutics will be administered in a manner compatible with the dosage formulation, and in such amount as is pharmacologically effective. The formulations are easily administered in a variety of dosage forms, such as the type of injectable solutions described above, but drug release capsules and the like can also be employed.

[00143] In this context, the quantity of active ingredient and volume of composition to be administered depends on the host animal to be treated. Precise amounts of active compound required for administration depend on the judgment of the practitioner and are peculiar to each individual.

[00144] A minimal volume of a composition required to disperse the active compounds is typically utilized. Suitable regimes for administration are also variable, but would be typified by initially administering the compound and monitoring the results and then giving further controlled doses at further intervals.

[00145] For instance, for oral administration in the form of a tablet or capsule (*e.g.*, a gelatin capsule), the active drug component can be combined with an oral, non-toxic pharmaceutically acceptable inert carrier such as ethanol, glycerol, water and the like. Moreover, when desired or necessary, suitable binders, lubricants, disintegrating agents and coloring agents can also be incorporated into the mixture. Suitable binders include starch, magnesium aluminum silicate, starch paste, gelatin, methylcellulose, sodium carboxymethylcellulose and/or polyvinylpyrrolidone, natural sugars such as glucose or beta-lactose, corn sweeteners, natural and synthetic gums such as acacia, tragacanth or sodium alginate, polyethylene glycol, waxes and the like. Lubricants used in these dosage forms include sodium oleate, sodium stearate, magnesium stearate, sodium benzoate, sodium acetate, sodium chloride, silica, talcum, stearic acid, its magnesium or calcium salt and/or polyethyleneglycol and the like. Disintegrators include, without limitation, starch, methyl cellulose, agar, bentonite, xanthan gum starches, agar, alginic acid or its sodium salt, or effervescent mixtures, and the like. Diluents, include, *e.g.*, lactose, dextrose, sucrose, mannitol, sorbitol, cellulose and/or glycine.

[00146] Injectable compositions are preferably aqueous isotonic solutions or suspensions, and suppositories are advantageously prepared from fatty emulsions or suspensions. The compositions may be sterilized and/or contain adjuvants, such as preserving, stabilizing, wetting or emulsifying agents, solution promoters, salts for regulating the osmotic pressure and/or buffers. In addition, they may also contain other therapeutically valuable substances. The compositions are prepared according to conventional mixing, granulating or coating methods, respectively, and typically contain about 0.1 to 75%, preferably about 1 to 50%, of the active ingredient.

[00147] The compounds of the invention can also be administered in such oral dosage forms as timed release and sustained release tablets or capsules, pills, powders, granules, elixirs, tinctures, suspensions, syrups and emulsions.

[00148] Liquid, particularly injectable compositions can, for example, be prepared by dissolving, dispersing, etc. The active compound is dissolved in or mixed with a pharmaceutically pure solvent such as, for example, water, saline, aqueous dextrose, glycerol, ethanol, and the like, to thereby form the injectable solution or suspension. Additionally, solid forms suitable for dissolving in liquid prior to injection can be formulated.

[00149] The compounds of the present invention can be administered in intravenous (both bolus and infusion), intraperitoneal, subcutaneous or intramuscular form, all using forms well known to those of ordinary skill in the pharmaceutical arts. Injectables can be prepared in conventional forms, either as liquid solutions or suspensions.

[00150] Parenteral injectable administration is generally used for subcutaneous, intramuscular or intravenous injections and infusions. Additionally, one approach for parenteral administration employs the implantation of a slow-release or sustained-released systems, which assures that a constant level of dosage is maintained, according to U.S. Pat. No. 3,710,795, incorporated herein by reference.

[00151] Furthermore, preferred compounds for the present invention can be administered in intranasal form via topical use of suitable intranasal vehicles, inhalants, or via transdermal routes, using those forms of transdermal skin patches well known to those of ordinary skill in that art. To be administered in the form of a transdermal delivery system, the dosage administration will, of course, be continuous rather than intermittent throughout the dosage regimen. Other preferred topical preparations include creams, ointments, lotions, aerosol sprays and gels, wherein the concentration of active ingredient would typically range from 0.01% to 15%, w/w or w/v.

[00152] For solid compositions, excipients include pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharin, talcum, cellulose, glucose, sucrose, magnesium carbonate, and the like may be used. The active compound defined above, may be also formulated as suppositories using for example, polyalkylene glycols, for example, propylene glycol, as the carrier. In some embodiments, suppositories are advantageously prepared from fatty emulsions or suspensions.

[00153] The compounds of the present invention can also be administered in the form of liposome delivery systems, such as small unilamellar vesicles, large unilamellar vesicles and multilamellar vesicles. Liposomes can be formed from a variety of phospholipids, containing cholesterol, stearylamine or phosphatidylcholines. In some embodiments, a film of lipid components is hydrated with an aqueous solution of drug to a form lipid layer encapsulating the drug, as described in U.S. Pat. No. 5,262,564. For example, the aptamer molecules described herein can be provided as a complex with a lipophilic compound or non-immunogenic, high

molecular weight compound constructed using methods known in the art. An example of nucleic-acid associated complexes is provided in U.S. Patent No. 6,011,020.

[00154] The compounds of the present invention may also be coupled with soluble polymers as targetable drug carriers. Such polymers can include polyvinylpyrrolidone, pyran copolymer, polyhydroxypropyl-methacrylamide-phenol, polyhydroxyethylaspanamidephenol, or polyethyleneoxidepolylysine substituted with palmitoyl residues. Furthermore, the compounds of the present invention may be coupled to a class of biodegradable polymers useful in achieving controlled release of a drug, for example, polylactic acid, polyepsilon caprolactone, polyhydroxy butyric acid, polyorthoesters, polyacetals, polydihydropyrans, polycyanoacrylates and cross-linked or amphipathic block copolymers of hydrogels.

[00155] If desired, the pharmaceutical composition to be administered may also contain minor amounts of non-toxic auxiliary substances such as wetting or emulsifying agents, pH buffering agents, and other substances such as for example, sodium acetate, and triethanolamine oleate.

[00156] The dosage regimen utilizing the aptamers is selected in accordance with a variety of factors including type, species, age, weight, sex and medical condition of the patient; the severity of the condition to be treated; the route of administration; the renal and hepatic function of the patient; and the particular aptamer or salt thereof employed. An ordinarily skilled physician or veterinarian can readily determine and prescribe the effective amount of the drug required to prevent, counter or arrest the progress of the condition.

[00157] Oral dosages of the present invention, when used for the indicated effects, will range between about 0.05 to 5000 mg/day orally. The compositions are preferably provided in the form of scored tablets containing 0.5, 1.0, 2.5, 5.0, 10.0, 15.0, 25.0, 50.0, 100.0, 250.0, 500.0 and 1000.0 mg of active ingredient. Effective plasma levels of the compounds of the present invention range from 0.002 mg/mL to 50 mg/mL.

[00158] Compounds of the present invention may be administered in a single daily dose, or the total daily dosage may be administered in divided doses of two, three or four times daily.

Modulation of pharmacokinetics and biodistribution of aptamer therapeutics

[00159] It is important that the pharmacokinetic properties for all oligonucleotide-based therapeutics, including aptamers, be tailored to match the desired pharmaceutical application.

While aptamers directed against extracellular targets do not suffer from difficulties associated with intracellular delivery (as is the case with antisense and RNAi-based therapeutics), such aptamers must still be able to be distributed to target organs and tissues, and remain in the body (unmodified) for a period of time consistent with the desired dosing regimen.

[00160] Thus, the present invention provides materials and methods to affect the pharmacokinetics of aptamer compositions, and, in particular, the ability to tune aptamer pharmacokinetics. The tunability of (*i.e.*, the ability to modulate) aptamer pharmacokinetics is achieved through conjugation of modifying moieties (*e.g.*, PEG polymers) to the aptamer and/or the incorporation of modified nucleotides (*e.g.*, 2'-fluoro or 2'-O-methyl) to alter the chemical composition of the nucleic acid. The ability to tune aptamer pharmacokinetics is used in the improvement of existing therapeutic applications, or alternatively, in the development of new therapeutic applications. For example, in some therapeutic applications, *e.g.*, in anti-neoplastic or acute care settings where rapid drug clearance or turn-off may be desired, it is desirable to decrease the residence times of aptamers in the circulation. Alternatively, in other therapeutic applications, *e.g.*, maintenance therapies where systemic circulation of a therapeutic is desired, it may be desirable to increase the residence times of aptamers in circulation.

[00161] In addition, the tunability of aptamer pharmacokinetics is used to modify the biodistribution of an aptamer therapeutic in a subject. For example, in some therapeutic applications, it may be desirable to alter the biodistribution of an aptamer therapeutic in an effort to target a particular type of tissue or a specific organ (or set of organs). In these applications, the aptamer therapeutic preferentially accumulates in a specific tissue or organ(s). In other therapeutic applications, it may be desirable to target tissues displaying a cellular marker or a symptom associated with a given disease, cellular injury or other abnormal pathology, such that the aptamer therapeutic preferentially accumulates in the affected tissue. For example, as described in copending provisional application United States Serial No. 60/550790, filed on March 5, 2004, and entitled "Controlled Modulation of the Pharmacokinetics and Biodistribution of Aptamer Therapeutics) , PEGylation of an aptamer therapeutic (*e.g.*, PEGylation with a 20 kDa PEG polymer) is used to target inflamed tissues, such that the PEGylated aptamer therapeutic preferentially accumulates in inflamed tissue.

[00162] To determine the pharmacokinetic and biodistribution profiles of aptamer therapeutics (*e.g.*, aptamer conjugates or aptamers having altered chemistries, such as modified nucleotides) a variety of parameters are monitored. Such parameters include, for example, the half-life ($t_{1/2}$), the plasma clearance (Cl), the volume of distribution (Vss), the area under the concentration-time curve (AUC), maximum observed serum or plasma concentration (C_{max}), and the mean residence time (MRT) of an aptamer composition. As used herein, the term "AUC" refers to the area under the plot of the plasma concentration of an aptamer therapeutic versus the time after aptamer administration. The AUC value is used to estimate the bioavailability (*i.e.*, the percentage of administered aptamer therapeutic in the circulation after aptamer administration) and/or total clearance (Cl) (*i.e.*, the rate at which the aptamer therapeutic is removed from circulation) of a given aptamer therapeutic. The volume of distribution relates the plasma concentration of an aptamer therapeutic to the amount of aptamer present in the body. The larger the Vss, the more an aptamer is found outside of the plasma (*i.e.*, the more extravasation).

The present invention provides materials and methods to modulate, in a controlled manner, the pharmacokinetics and biodistribution of stabilized aptamer compositions *in vivo* by conjugating an aptamer to a modulating moiety such as a small molecule, peptide, or polymer terminal group, or by incorporating modified nucleotides into an aptamer. As described herein, conjugation of a modifying moiety and/or altering nucleotide(s) chemical composition alter fundamental aspects of aptamer residence time in circulation and distribution to tissues.

[00163] In addition to clearance by nucleases, oligonucleotide therapeutics are subject to elimination *via* renal filtration. As such, a nuclease-resistant oligonucleotide administered intravenously typically exhibits an *in vivo* half-life of <10 min, unless filtration can be blocked. This can be accomplished by either facilitating rapid distribution out of the blood stream into tissues or by increasing the apparent molecular weight of the oligonucleotide above the effective size cut-off for the glomerulus. Conjugation of small therapeutics to a PEG polymer (PEGylation), described below, can dramatically lengthen residence times of aptamers in circulation, thereby decreasing dosing frequency and enhancing effectiveness against vascular targets.

[00164] Aptamers can be conjugated to a variety of modifying moieties, such as high molecular weight polymers, *e.g.*, PEG; peptides, *e.g.*, Tat (a 13-amino acid fragment of the HIV Tat protein (Vives, *et al.* (1997), J. Biol. Chem. 272(25): 16010-7)), Ant (a 16-amino acid sequence derived from the third helix of the Drosophila antennapedia homeotic protein (Pietersz, *et al.* (2001), Vaccine 19(11-12): 1397-405)) and Arg_7 (a short, positively charged cell-permeating peptides composed of polyarginine (Arg_7) (Rothbard, *et al.* (2000), Nat. Med. 6(11): 1253-7; Rothbard, J *et al.* (2002), J. Med. Chem. 45(17): 3612-8)); and small molecules, *e.g.*, lipophilic compounds such as cholesterol. Among the various conjugates described herein, *in vivo* properties of aptamers are altered most profoundly by complexation with PEG groups. For example, complexation of a mixed 2'F and 2'-OMe modified aptamer therapeutic with a 20 kDa PEG polymer hinders renal filtration and promotes aptamer distribution to both healthy and inflamed tissues. Furthermore, the 20 kDa PEG polymer-aptamer conjugate proves nearly as effective as a 40 kDa PEG polymer in preventing renal filtration of aptamers. While one effect of PEGylation is on aptamer clearance, the prolonged systemic exposure afforded by presence of the 20 kDa moiety also facilitates distribution of aptamer to tissues, particularly those of highly perfused organs and those at the site of inflammation. The aptamer-20 kDa PEG polymer conjugate directs aptamer distribution to the site of inflammation, such that the PEGylated aptamer preferentially accumulates in inflamed tissue. In some instances, the 20 kDa PEGylated aptamer conjugate is able to access the interior of cells, such as, for example, kidney cells.

[00165] Modified nucleotides can also be used to modulate the plasma clearance of aptamers. For example, an unconjugated aptamer which incorporates both 2'-F and 2'-OMe stabilizing chemistries, which is typical of current generation aptamers as it exhibits a high degree of nuclease stability *in vitro* and *in vivo,* displays rapid loss from plasma (*i.e.*, rapid plasma clearance) and a rapid distribution into tissues, primarily into the kidney, when compared to unmodified aptamer.

PEG-Derivatized Nucleic Acids.

[00166] As described above, derivatization of nucleic acids with high molecular weight non-immunogenic polymers has the potential to alter the pharmacokinetic and pharmacodynamic

properties of nucleic acids making them more effective therapeutic agents. Favorable changes in activity can include increased resistance to degradation by nucleases, decreased filtration through the kidneys, decreased exposure to the immune system, and altered distribution of the therapeutic through the body.

[00167] The aptamer compositions of the invention may be derivatized with polyalkylene glycol (PAG) moieties. Examples of PAG-derivatized nucleic acids are found in United States Patent Application Ser. No. 10/718,833, filed on November 21, 2003, which is herein incorporated by reference in its entirety. Typical polymers used in the invention include poly(ethylene glycol) (PEG), also known as poly(ethylene oxide) (PEO) and polypropylene glycol (including poly isopropylene glycol). Additionally, random or block copolymers of different alkylene oxides (*e.g.*, ethylene oxide and propylene oxide) can be used in many applications. In its most common form, a polyalkylene glycol, such as PEG, is a linear polymer terminated at each end with hydroxyl groups: $HO\text{-}CH_2CH_2O\text{-}(CH_2CH_2O)_n\text{-}CH_2CH_2\text{-}OH$. This polymer, alpha-, omega-dihydroxylpoly(ethylene glycol), can also be represented as HO-PEG-OH, where it is understood that the —PEG- symbol represents the following structural unit: $\text{-}CH_2CH_2O\text{-}(CH_2CH_2O)_n\text{-}CH_2CH_2\text{-}$ where n typically ranges from about 4 to about 10,000.

[00168] As shown, the PEG molecule is di-functional and is sometimes referred to as "PEG diol." The terminal portions of the PEG molecule are relatively non-reactive hydroxyl moieties, the –OH groups, that can be activated, or converted to functional moieties, for attachment of the PEG to other compounds at reactive sites on the compound. Such activated PEG diols are referred to herein as bi-activated PEGs. For example, the terminal moieties of PEG diol have been functionalized as active carbonate ester for selective reaction with amino moieties by substitution of the relatively nonreactive hydroxyl moieties, -OH, with succinimidyl active ester moieties from N-hydroxy succinimide.

[00169] In many applications, it is desirable to cap the PEG molecule on one end with an essentially non-reactive moiety so that the PEG molecule is mono-functional (or mono-activated). In the case of protein therapeutics which generally display multiple reaction sites for activated PEGs, bi-functional activated PEGs lead to extensive cross-linking, yielding poorly functional aggregates. To generate mono-activated PEGs, one hydroxyl moiety on the terminus of the PEG diol molecule typically is substituted with non-reactive methoxy end moiety, -

OCH_3. The other, un-capped terminus of the PEG molecule typically is converted to a reactive end moiety that can be activated for attachment at a reactive site on a surface or a molecule such as a protein.

[00170] PAGs are polymers which typically have the properties of solubility in water and in many organic solvents, lack of toxicity, and lack of immunogenicity. One use of PAGs is to covalently attach the polymer to insoluble molecules to make the resulting PAG-molecule "conjugate" soluble. For example, it has been shown that the water-insoluble drug paclitaxel, when coupled to PEG, becomes water-soluble. Greenwald, *et al., J. Org. Chem.*, 60:331-336 (1995). PAG conjugates are often used not only to enhance solubility and stability but also to prolong the blood circulation half-life of molecules.

[00171] Polyalkylated compounds of the invention are typically between 5 and 80 kD in size however any size can be used, the choice dependent on the aptamer and application. Other PAG compounds of the invention are between 10 and 80 kD in size. Still other PAG compounds of the invention are between 10 and 60 kD in size. For example, a PAG polymer may be at least 10, 20, 30, 40, 50, 60, or 80 kD in size. Such polymers can be linear or branched.

[00172] In contrast to biologically-expressed protein therapeutics, nucleic acid therapeutics are typically chemically synthesized from activated monomer nucleotides. PEG-nucleic acid conjugates may be prepared by incorporating the PEG using the same iterative monomer synthesis. For example, PEGs activated by conversion to a phosphoramidite form can be incorporated into solid-phase oligonucleotide synthesis. Alternatively, oligonucleotide synthesis can be completed with site-specific incorporation of a reactive PEG attachment site. Most commonly this has been accomplished by addition of a free primary amine at the 5'-terminus (incorporated using a modifier phosphoramidite in the last coupling step of solid phase synthesis). Using this approach, a reactive PEG (*e.g.*, one which is activated so that it will react and form a bond with an amine) is combined with the purified oligonucleotide and the coupling reaction is carried out in solution.

[00173] The ability of PEG conjugation to alter the biodistribution of a therapeutic is related to a number of factors including the apparent size (*e.g.*, as measured in terms of hydrodynamic radius) of the conjugate. Larger conjugates (>10kDa) are known to more effectively block

filtration via the kidney and to consequently increase the serum half-life of small macromolecules (*e.g.*, peptides, antisense oligonucleotides). The ability of PEG conjugates to block filtration has been shown to increase with PEG size up to approximately 50 kDa (further increases have minimal beneficial effect as half life becomes defined by macrophage-mediated metabolism rather than elimination via the kidneys).

[00174] Production of high molecular weight PEGs (>10 kDa) can be difficult, inefficient, and expensive. As a route towards the synthesis of high molecular weight PEG-nucleic acid conjugates, previous work has been focused towards the generation of higher molecular weight activated PEGs. One method for generating such molecules involves the formation of a branched activated PEG in which two or more PEGs are attached to a central core carrying the activated group. The terminal portions of these higher molecular weight PEG molecules, *i.e.*, the relatively non-reactive hydroxyl (–OH) moieties, can be activated, or converted to functional moieties, for attachment of one or more of the PEGs to other compounds at reactive sites on the compound. Branched activated PEGs will have more than two termini, and in cases where two or more termini have been activated, such activated higher molecular weight PEG molecules are referred to herein as, multi-activated PEGs. In some cases, not all termini in a branch PEG molecule are activated. In cases where any two termini of a branch PEG molecule are activated, such PEG molecules are referred to as bi-activated PEGs. In some cases where only one terminus in a branch PEG molecule is activated, such PEG molecules are referred to as mono-activated. As an example of this approach, activated PEG prepared by the attachment of two monomethoxy PEGs to a lysine core which is subsequently activated for reaction has been described (Harris *et al.*, Nature, vol.2: 214-221, 2003).

[00175] The present invention provides another cost effective route to the synthesis of high molecular weight PEG-nucleic acid (preferably, aptamer) conjugates including multiply pegylated nucleic acids (as illustrated, *e.g.*, in Fig. 6). The present invention also encompasses PEG-linked multimeric oligonucleotides, *e.g.*, dimerized aptamers (as also illustrated, *e.g.*, in Fig. 6).

[00176] High molecular weight compositions of the invention include those having a molecular weight of at least 10 kD. Compositions typically have a molecular weight between

10 and 80 kD in size. High molecular weight compositions of the invention are at least 10, 20, 30, 40, 50, 60, or 80 kD in size.

[00177] A stabilizing moiety is a molecule, or portion of a molecule, which improves pharmacokinetic and pharmacodynamic properties of the high molecular weight aptamer compositions of the invention. In some cases, a stabilizing moiety is a molecule or portion of a molecule which brings two or more aptamers, or aptamer domains, into proximity, or provides decreased overall rotational freedom of the high molecular weight aptamer compositions of the invention. A stabilizing moiety can be a polyalkylene glycol, such a polyethylene glycol, which can be linear or branched, a homopolymer or a heteropolymer. Other stabilizing moieties include polymers such as peptide nucleic acids (PNA). Oligonucleotides can also be stabilizing moieties; such oligonucleotides can include modified nucleotides, and/or modified linkages, such as phosphothioates. A stabilizing moiety can be an integral part of an aptamer composition, *i.e.*, it is covalently bonded to the aptamer. Alternatively, the stabilizing moiety can associate with the aptamer composition non-covalently, such as *viá* hydrogen bonding or hybridization interactions between two oligonucleotides.

PEG-mediated Dimerization of Aptamers.

[00178] Dimerization *via* a bi-functional PEG offers multiple potential benefits including (1) increased affinity in binding to dimeric targets, (2) increased avidity and decreased dissociation rate in binding to all targets, and (3) increased effective molecular weight with corresponding increased resistance to clearance via filtration.

[00179] Compositions of the invention include high molecular weight aptamer compositions in which two or more aptamers are covalently conjugated to at least one polyalkylene glycol moiety. The polyalkylene glycol moieties serve as stabilizing moieties. In compositions where a polyalkylene glycol moiety is covalently bound at either end to an aptamer, such that the polyalkylene glycol joins the aptamers together in one molecule, the polyalkylene glycol is said to be a linking moiety. In such compositions, the primary structure of the covalent molecule includes the linear arrangement aptamer-PAG-aptamer. One example is a composition having the primary structure aptamer-PEG-aptamer.

[00180] To produce the nucleic acid—PEG—nucleic acid conjugate, the nucleic acid is originally synthesized such that it bears a single reactive site (*e.g.*, it is mono-activated). In a preferred embodiment, this reactive site is an amino group introduced at the 5'-terminus by addition of a modifier phosphoramidite as the last step in solid phase synthesis of the oligonucleotide. Following deprotection and purification of the modified oligonucleotide, it is reconstituted at high concentration in a solution that minimizes spontaneous hydrolysis of the activated PEG. In a preferred embodiment, the concentration of oligonucleotide is 1 mM and the reconstituted solution contains 200 mM $NaHCO_3$-buffer, pH 8.3. Synthesis of the conjugate is initiated by slow, step-wise addition of highly purified bi-functional PEG. In a preferred embodiment, the PEG diol is activated at both ends (bi-activated) by derivatization with succinimidyl propionate. Following reaction, the PEG-nucleic acid conjugate is purified by gel electrophoresis or liquid chromatography to separate fully-, partially-, and un-conjugated species. Multiple PAG molecules concatenated (*e.g.*, as random or block copolymers) or smaller PAG chains can be linked to achieve various lengths (or molecular weights). Non-PAG linkers can be used between PAG chains of varying lengths.

PAG-derivatization of a reactive nucleic acid.

[00181] High molecular weight PAG-nucleic acid-PAG conjugates can be prepared by reaction of a mono-functional activated PEG with a nucleic acid containing more than one reactive site. In one embodiment, the nucleic acid is bi-reactive, or bi-activated, and contains two reactive sites: a 5'-amino group and a 3'-amino group introduced into the oligonucleotide through conventional phosphoramidite synthesis, for example: 3'-5'-di-PEGylation as illustrated in Figure 7. In alternative embodiments, reactive sites can be introduced at internal positions, using for example, the 5-position of pyrimidines, the 8-position of purines, or the 2'-position of ribose as sites for attachment of primary amines. In such embodiments, the nucleic acid can have several activated or reactive sites and is said to be multiply activated. Following synthesis and purification, the modified oligonucleotide is combined with the mono-activated PEG under conditions that promote selective reaction with the oligonucleotide reactive sites while minimizing spontaneous hydrolysis. In the preferred embodiment, monomethoxy-PEG is activated with succinimidyl propionate and the coupled reaction is carried out at pH 8.3. To

drive synthesis of the bi-substituted PEG, stoichiometric excess PEG is provided relative to the oligonucleotide. Following reaction, the PEG-nucleic acid conjugate is purified by gel electrophoresis or liquid chromatography to separate fully-, partially-, and un-conjugated species. Figure 6 illustrates the two strategies for synthesizing PEGylated nucleic acid aptamers.

[00182] The linking domains can also have one or more polyalkylene glycol moieties attached thereto. Such PAGs can be of varying lengths and may be used in appropriate combinations to achieve the desired molecular weight of the composition.

[00183] The effect of a particular linker can be influenced by both its chemical composition and length. A linker that is too long, too short, or forms unfavorable steric and/or ionic interactions with the target will preclude the formation of complex between aptamer and target. A linker, which is longer than necessary to span the distance between nucleic acids, may reduce binding stability by diminishing the effective concentration of the ligand. Thus, it is often necessary to optimize linker compositions and lengths in order to maximize the affinity of an aptamer to a target.

Multivalent Aptamer Design Strategies

[00184] One potential solution to the inherent problem of gaining regulatory approval for combination therapies is the use of multivalent aptamers, *i.e.*, aptamers that are able to bind to more than one of the targets that are implicated for a particular indication (or conceivably different indications). Aptamers such as these can inhibit multiple proteins (or other targets) and therefore provide a single compound that acts in a manner that is substantially equivalent to a combination of compounds. Multi-functional aptamers can be engineered, *e.g.*, from combinations of known aptamers. These multivalent aptamers can be shown to bind to multiple targets, and can be generated either directly by solid-phase chemical synthesis, or by transcription from a corresponding DNA template. Examples of such multi-functional aptamers include aptamers capable of binding to TGFβ1 and TGFβ2 and/or PDGF for anti-scarring indications, and VEGF and PDGF for cancer indications.

[00185] The present invention provides a generalizable design strategy for the generation of oligonucleotide aptamers that bind to more than one target. The present invention also provides

oligonucleotide aptamers that bind to more than one target and can separately inhibit multiple targets implicated in a disease or disorder, and thus act as if combinations of therapeutics were being used. Since multivalent aptamers are single compounds, their regulatory approval can be significantly easier than is the case for combination therapies in which multiple compounds need to be separately assessed for their toxicology. And, for combination therapies, the combination needs to be demonstrably more efficacious than the individual components by themselves, which means an additional burden of proof.

[00186] The ability of aptamers to bind to a variety of targets including but not limited to proteins, peptides, and small molecules, can be an advantage of this class of therapeutics over many therapeutic agents currently used. Aptamers are able to fold into defined three-dimensional conformations and bind to molecular targets with great specificity and affinity. Of particular interest is the ability of aptamers to bind to proteins, many of which are implicated in disease states, and thus inhibit their pathogenesis in those disease states. This ability of aptamers is exploited by the methods of the present invention to produce a single oligonucleotide aptamer therapeutic that can bind to several molecular targets simultaneously or to various parts of a single target, thus enhancing its therapeutic effectiveness and potency.

[00187] In one embodiment, in order to design multivalent aptamers from previously identified aptamers (or regions of aptamers) it is important to conjoin the individual aptamers via regions of the individual aptamer that do not make contact with the target. This can typically be accomplished by identifying regions of the secondary structure which tolerate substitution of individual nucleotides at most or all positions. If structural units are required, such as a stem, then these can be preserved in the final design. Additionally, it is important that the structural integrity of each of the individual aptamers is preserved in the final folded structure. This can most easily be achieved by predicting the secondary structures of the original aptamer sequences using an algorithm such as mfold, and then ensuring that these predicted secondary structures are preserved, according to the same algorithm, when they are part of the conjoined structure. The general mfold algorithm for determining multiple optimal and suboptimal secondary structures is described by the author of the program, Dr. Michael Zuker (Science 244, 48-52 (1989)). A description of the folding parameters used in the algorithm is presented in Jaeger, Turner, and Zuker , Proc. Natl. Acad. Sci. USA, 86, 7706-7710 (1989) (see also M.

Zuker, *et al.*, Algorithms and Thermodynamics for RNA Secondary Structure Prediction: A Practical Guide in RNA Biochemistry and Biotechnology, J. Barciszewski and B.F.C. Clark, eds., NATO ASI Series, Kluwer Academic Publishers, (1999)). Other programs that can be used according to the methods of the present invention to obtain secondary structures of nucleic acid sequences include, without limitation, RNAStructure (Mathews, D.H.; Sabina, J.; Zuker, M.; and Turner, D.H., "Expanded Sequence Dependence of Thermodynamic Parameters Improves Prediction of RNA Secondary Structure," Journal of Molecular Biology, 288, 911-940(1999), and RnaViz (Nucleic Acids Res., Nov. 15; 25(22):4679-84 (1997).

[00188] Having determined the secondary structural motifs and units of the component aptamers, these are combined into a single oligonucleotide that folds in a manner that preserves the functionalities of each of the constituent aptamers. As a result, multivalent aptamers are capable of binding to multiple targets, and can be generated either directly by solid phase chemical synthesis, or by transcription from a corresponding DNA template.

[00189] The multivalent aptamers produced by the methods of the present invention are able to bind to more than one target implicated in a disease state, or alternatively, to targets implicated in distinct but related diseases states or indications. The multivalent aptamers can inhibit multiple proteins and therefore a single compound to act in a manner that is substantially equivalent to, or potentially more potent than, a combination of compounds.

[00190] The methods of the present invention optionally provide that an aptamer sequence be minimized prior to conjoining it with other aptamers so as to keep the molecular weight and size of the resulting multivalent aptamer to a minimum.

[00191] The present invention as described above provides methods to join a plurality of aptamers which have been identified by the SELEX™ process described above. In one embodiment, the methods of the present invention provide for *de novo* selection of an aptamer that has binding sites for multiple targets with SELEX™. In another embodiment, the methods of the present invention provide for the *de novo* selection of at least one binding site and not the others by, *e.g.*, appending a randomized region of oligonucleotides to an aptamer and performing SELEX against a target not recognized by the starting aptamer. In another embodiment, the present invention provides multivalent aptamers, *e.g.*, bivalent aptamers that bind a second target only after a first target is bound.

[00192] In another embodiment, the methods of the present invention provide for chemical linking of aptamers with a linker, *e.g.*, polyethylene glycol (PEG). In another embodiment, the methods of the present invention provide for the linking of the aptamer portions of a multivalent aptamer with an oligonucleotide linker that has been rationally designed or selected from randomized regions. For example, disclosed herein are splinted and non-splinted multivalent (*e.g.*, bivalent or dimerized) aptamers. The splinted multivalent aptamers (illustrated, *e.g.*, in Fig. 8A) are comprised of two oligonucleotides. The first oligonucleotide is comprised of two or more aptamer domains (*e.g.*, target binding domains), which can be previously identified aptamers or regions of previously identified aptamers joined by a single-stranded linker domain. The second oliogonucleotide (or "splint oligonucleotide") is complementary and binds to a portion of the linker domain of the first oligonucleotide. The oligonucleotide splint can have a nucleic acid sequence that includes at least 40 nucleotides. When bound to the linking domain, the oligonucleotide splint preferably has at least twenty nucleotides hybridized to the linking domain. The oligonucleotide splint can also have one or more polyalkylene glycol moieties attached thereto. In some splinted, multivalent aptamer compositions, at least one of the two or more aptamer domains is in an unbound state (*i.e.*, not bound to a specific target). Binding of the splint oligonucleotide, which is preferably DNA, to the first oligonucleotide is believed to increase stability by (1) providing some rigidity to the first oligonucleotide, (2) preventing the single-stranded region from interacting with the target binding regions, and/or (3) reducing rotational freedom.

[00193] Non-splinted multivalent aptamers (illustrated, *e.g.*, in Fig. 10) are comprised of a single oligonucleotide comprising two target binding domains (*e.g.*, previously identified aptamers or regions of previously identified aptamers) joined by a single-stranded linker domain. The linking domain of these non-splinted multivalent aptamers can have one ore more polyalkylene glycol moieties attached thereto. Such PAGs can be of varying lengths and may be used in appropriate combinations to achieve the desired molecular weight of the composition.

[00194] Chelating ligands, typically multivalent (*e.g.*, bivalent), are known to be more stable than monodentate, or monovalent, ligands. In the present invention, an oligonucleotide linker is used to link multiple aptamers to achieve a chelating effect with a bidentate or multivalent

aptamer. The linker region can be used with or without a "splint oligonucleotide" to further stabilize the construct. The composition of the oligonucleotide linker can be of a heterogeneous sequence or it can be a poly U/C or poly A/G linker of various sequences and/or lengths. The effect of the various linker sequences and composition has desirable effects on aptamer ligand properties as described in Example 5 and Example 6.

[00195] Multivalent aptamers have improved pharmacokinetic and/or pharmacodynamic properties relative to monovalent aptamers. Both enthalpic and entropic effects contribute to the enhanced affinity of chelator-like multivalent aptamers. The enthalpic gain results from the several additional interactions formed between the bivalent ligand and its target. The entropic gain, in part, reflects the reduced entropic penalty associated with the formation of a 1:1 complex between bivalent ligand and target as compared to the binding of two monovalent ligands to the same target. Additionally, the "effective concentration" of the ligand is increased. For example, when a monomeric ligand dissociates from its target, it is released into bulk solution; however, when one of the liganding moieties of a chelate dissociates, its movement is constrained to within the proximity of the target by tethering to the bound cognate moiety.

[00196] The effect of a particular linker sequence can be influenced by both its chemical composition and length. A linker that is too short will clearly preclude the formation of a chelate. However, a linker that forms unfavorable steric and/or ionic interactions with the target will also negate the stabilizing effects of chelation. On the other hand, lengthening of the linker, beyond that necessary to span the distance between binding sites may reduce binding stability by diminishing the effective concentration of the ligand. Thus, it is often necessary to optimize linker compositions and lengths in order to maximize the affinity of a chelating ligand. Nevertheless, preliminary measurements with bidentate aptamers specific to two cytokine homodimers (PDGF and TGFβ2), as described in Example 5 and Example 6, indicate that linking aptamers has desirable effects on aptamer binding properties. The derivatized aptamers having improved pharmacokinetic and pharmacodynamic properties of the present invention can be initially obtained by the "Systematic Evolution of Ligands by EXponential Enrichment" ("SELEX™") method as described above.

[00197] All publications and patent documents cited herein are incorporated herein by reference as if each such publication or document was specifically and individually indicated to be incorporated herein by reference. Citation of publications and patent documents is not intended as an admission that any is pertinent prior art, nor does it constitute any admission as to the contents or date of the same. The invention having now been described by way of written description, those of skill in the art will recognize that the invention can be practiced in a variety of embodiments and that the foregoing description and examples below are for purposes of illustration and not limitation of the claims that follow.

EXAMPLES

EXAMPLE 1 VEGF and PDGF multivalent aptamer

[00198] The methods of the present invention were applied to generate an aptamer having binding specificity to PDGF BB and to VEGF. This multi-functional aptamer was generated by joining two aptamers individually identified using SELEX – one (ARC 245) which recognized VEGF but not PDGF (see Figure 2(A)) and one (ARC 126) which recognized PDGF but not VEGF (see Figure 2(B)). A schematic of the structure and sequence of the multivalent aptamer capable of binding to PDGF and VEGF resulting from this combination is shown in Figure 2(C) (TK.131.012.A (SEQ ID NO:3)). Figures 3(B) and (C) show binding plots of multivalent aptamers with (A) PDGF BB and (B) VEGF binding affinity.

[00199] ARC245 (SEQ ID NO:1) is a fully 2'O methylated (2'-OMe) aptamer with binding specificity to VEGF with a K_D of about 2 nM. ARC126 (SEQ ID NO:2) is an all DNA aptamer with binding specificity to PDGF with a K_D of about 0.4 nM.

[00200] Binding data for the constituent aptamers and the multivalent aptamer was collected by dot-blot assays in which radio-labeled aptamer is incubated with protein target and then forced through a sandwich of nitrocellulose over nylon. Protein-associated radio-label is captured on the nitrocellulose membrane while the balance of the radio-label is captured on the nylon membrane. The radio-label data is collected on a phosphorimaging plate. This data is then used to calculate the binding coefficients. This multivalent aptamer (SEQ ID NO:3) was made synthetically.

[00201] The multivalent aptamer created by combining the structural motifs according to the methods of the present invention shows a K_D of about 10 nM for PDGF and about 10 nM for VEGF.

[00202] SEQ ID NO:1 (ARC245, "m" indicates 2'-OMe nucleotides and "[3T]"refers to an inverted thymidine nucleotide that is attached to the 3'end of the oligonucleotide at the 3' position on the ribose sugar, thus the oligonucleotide has two 5'ends and is thus resistant to nucleases acting on the 3' hydroxyl end)
5′-mAmUmGmCmAmGmUmUmUmGmAmGmAmAmGmUmCmGmCmGmCmAmU-[3T]-3′

[00203] SEQ ID NO:2 (ARC126, "d" indicates unmodified deoxynucleotides,"[3T]" is as defined above and "f" indicates 2'F nucleotides).
5′-dCdAdGdGdCdTdAdCdGdCdGdTdAdGdAdGdCdAdTdCdAdTdGdAdTdCdCdTdG-[3T]-3′

[00204] SEQ ID NO:3 (TK.131.012.A, "m" indicates 2'-OMe nucleotides, "d" and "[3T]"are as defined above).
5′dCdAdGdGdCdTdAdCdGmAmUmGmCmAmGmUmUmUmGmAmGmAmAmGmUmCmGmCmGmCmAmUdCdGdTdAdGdAdGdCdAdTdCdAdGdAdAdAdTdGdAdTdCdCdTdG[3T]-3′

EXAMPLE 2 VEGF and PDGF multivalent aptamer

[00205] Figure 3(A) shows a schematic of the structure of a second multivalent aptamer capable of binding to VEGF and PDGF formed by combining ARC245 and ARC 126. As shown, in this VEGF-PDGF multivalent aptamer the first mA-mU pair of the stem of ARC245 was removed before joining ARC245 and ARC126. Figure 3(B) shows a binding curve of this multivalent aptamer (SEQ ID NO: 4) to (B) PDGF BB and (C) VEGF binding affinity.

[00206] ARC245 (SEQ ID NO:1) (entirely 2'OMe) is an aptamer with binding specificity to VEGF with a K_D of about 2 nM. ARC126 (SEQ ID NO:2) is entirely DNA with binding specificity to PDGF with a K_D of about 0.4 nM. The multivalent aptamer SEQ ID NO:4 created by combining the structural motifs according to the methods of the present invention shows a K_D of about 10 nM for PDGF and about 5 nM for VEGF. The multivalent aptamer of SEQ ID NO:4 was made synthetically.

[00207] SEQ ID NO:4 (TK.131.012.B, "m" and "[3T]" are as defined above).
5'-dCdAdGdGdCdTdAdCdGmUmGmCmAmGmUmUmUmGmAmGmAmAmGmUmCmGm-CmGmCmAdCdGdTdAdGdAdGdCdAdTdCdAdGdAdAdAdTdGdAdTdCdCdTdG-[3T]

EXAMPLE 3 TGFβ1 and TGFβ2 multivalent aptamer

[00208] ARC191, shown in Figure 4(A) (SEQ ID NO:5) (rRfY: ribo- A and G and fluoro- C and U nucleotides), has a K_D of about 3 nM for TGFβ1. ARC281, shown in Figure 4(B) (SEQ ID NO:6) (rRfY), has an IC_{50} in a cell based assay of about 40 nM and a K_D of about 500 pM for TGFβ2. The multivalent aptamer generated by combining ARC191 and ARC281 is shown in Figure 4(C) (SEQ ID NO:7). The multivalent aptamer was generated by combining ARC191 and ARC281 at a conserved stem structure, fusing them by generating a transcription template by PCR, and transcribing the template under rRfY conditions as described above. The resulting multivalent aptamer (SEQ ID NO:7) has a K_D of about 14 nM for TGFβ1 and a K_D of about 400 pM for TGFβ2.

[00209] Figure 4(A) shows a schematic of the secondary structure of ARC191 TGFβ1 aptamer and Figure 4(B) a schematic of the secondary structure of ARC281 TGFβ2 aptamer. Both were synthesized as antisense with a T7 promoter and primer sequence and a transcription template for PCR was generated and transcribed with ribo A and G and fluoro C and U nucleotides. Figure 4(C) shows a schematic of the secondary structure of an aptamer capable of binding to TGFβ1 and TGFβ2.

[00210] Figure 5(A) shows a binding plot of the resulting multivalent aptamer (SEQ ID NO:7) to TGFβ1 having a K_D of 14 nM and Figure 5(B) shows a binding plot of multivalent aptamer

(SEQ ID NO:7) to TGFβ2 with a K_D of 400 pM. ARC210 is used as control in the TGFβ1 and has identical sequence to ARC191 save for having an inverted T residue at the 3' end for improved stability.

[00211] SEQ ID NO:5 (ARC191: ribo A and G and fluoro C and U nucleotides)
5'-
mGmGmGfUGfCfCfUfUfUfUGfCfCfUmAmGmGfUfUmGfUmGmAfUfUfUmGfUmAmA-fCfCfUfUfCfUGfCfCfCmA-3'

[00212] SEQ ID NO:6 (ARC281: ribo A and G and fluoro C and U nucleotides, -[3T])
5'-AAGGAGfUAfUfUAfUAGAGfUAfUGfUAfUAGfCfUAfUAfCfCAfU-[3T]-3'

[00213] SEQ ID NO:7 (TK.131.020.A ribo A and G and fluoro C and U nucleotides)
5'-
GGGUGCCUUUUGCCUAACAGAGUAUUAUAGAGUAUGUAUAGCUAUACUGUUU-CUGCCCA-3'

EXAMPLE 4 PDGF, TGFβ1, and TGFβ2 multivalent aptamer

[00214] The methods of the present invention can be used to generate an aptamer having binding specificity to TGFβ1 and TGFβ2 and/or PDGF having potential therapeutic utility as an anti-scarring agent.

EXAMPLE 5 Bidentate PDGF Aptamers with an Oligonucleotide Splint Stabilizer

[00215] High molecular weight aptamer compositions capable of binding to platelet derived growth factor (PDGF) were produced using the following methods. A dimeric, or bidentate PDGF aptamer having the sequence shown in Figure 8(A) (SEQ ID NO:9) was synthesized using standard reagents (oligonucleotides supplied by IDT, Coralville, IA). As shown in Fig. 8(B), the enhanced affinity of the bidentate aptamer to its target (either PDGF BB or AB) was greatest at higher protein concentrations where the binding conditions were 25°C in 1X PBS with an RNA ligand concentration of < 10 pM. In addition, as shown in Figure 9, the use of a DNA splint complementary to the linker region as illustrated in Figure 8(A), had an enhancing

effect on the affinity of the bidentate aptamer ligand to PDGF-BB as shown in the plots of proportion of bidentate aptamer to PDGF-BB target with and without an oligonucleotide splint. This enhancement could reflect a splint-dependent reduction in rotational degrees of freedom within the linker region, leading to an increase in the effective concentration of the bidentate ligand. The addition of splint DNA to the monovalent aptamer to PDGF-BB had no effect on binding affinity (Figure 9).

[00216] SEQ ID NO:8 – splint oligonucleotide

5'-AAAGGAATTCTACGCCTCGAGTGCAGCCCAGGAACTATTT-3'

[00217] SEQ ID NO:9 -PDGF bidentate aptamer

5'-
TACTCAGGGCACTGCAAGCAATTGTGGTCCCAATGGGCTGAGTATGTGGTCTATG-
TCGTCGTTCGCTAGTAGTTCCTGGGCTGCACTCGAGGCGTAGAATTCCCCCGATGC
GCGCTGTTCTTACTCAGGGCACTGCAAGCAATTGTGGTCCCAATGGGCTGAGTAT-
3'

EXAMPLE 6 Bidentate TGFβ2 Aptamers with Homolymeric Oligonucleotide Linkers

[00218] High molecular weight aptamer compositions capable of binding to TGFβ2 were produced using the following methods. Several constructs of TGFβ2 bidentate aptamers based on a TGFβ2 aptamer having the sequence shown below in SEQ ID NO:10 were synthesized with poly U/C linkers of various lengths and sequence compositions as shown in Figure 10. When linking the aptamers a double helical extension at the 3' end of the aptamer was added to disrupt irrelevant conformers. Table 1 shows various spacer lengths and sequences that were used in the synthesis of the TGFβ2 bidentate aptamers.

[00219] SEQ ID NO:10 ("f" indicates modified nucleotides having a fluoro group at the 2' position)

5'-GGAGGfUfUAfUfUAfCAGAGfUfCfUGfUAfUAGfCfUGfUAfCfUfCfC-[3T]-3'

[00220] Table 1. Linker Sequences, N = length of oligonucleotide.

	N	sequence
SEQ ID NO:11	5	UUUUU
SEQ ID NO:12	10	UU UCCU UUUU
SEQ ID NO:13	20	UU $(UCCU)_3$CUUUUU
SEQ ID NO:14	30	UU $(UCCU)_6$UUUU
SEQ ID NO:15	40	U $(UCCU)_8$UCUUUUU
SEQ ID NO:16	50	U $(UCCU)_3$UU $(UCCU)_7$UC $(U)_5$

[00221] Figure 11 shows a binding plot showing the proportion of bidentate aptamer with various linker lengths and compositions and their effect on binding to TGFβ2. The effects of linkage length are <2-fold in K_D and are linker length dependent.

What is claimed is:

1. A multivalent aptamer comprising a single oligonucleotide having a sequence selected from the group consisting of SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:7, SEQ ID NO:9, and SEQ ID NO:10, where said aptamer has binding specificity to a plurality of targets.

2. The multivalent aptamer of Claim 1 wherein said binding specificities are to one or more targets selected from TGFβ1, TGFβ2, PDGF, and VEGF.

3. The multivalent aptamer of Claim 2 wherein said binding specificities are to the same target.

4. The multivalent aptamer of Claim 2 wherein said binding specificities are to different targets.

5. A method of generating a multivalent aptamer comprising the steps of:
(a) generating multiple aptamers by SELEX process having specificity to multiple targets;
(b) combining the structural motifs from each aptamer; and
(c) generating a single nucleic acid molecule incorporating the motifs from the multiple aptamers into said single multivalent aptamer nucleic acid molecule.

6. The method of Claim 5 further comprising the step of identifying minimal structural motifs or sequences that are responsible for each aptamer's binding specificity.

7. The method of Claim 5 wherein the single nucleic acid molecule is generated synthetically.

8. The method of Claim 5 wherein the single nucleic acid molecule is generated enzymatically.

9. A method of treating a disease in a subject comprising the step of administering to the subject a therapeutically effective amount of an aptamer therapeutic having binding specificity to multiple targets.

10. A therapeutic composition for treating disease in a subject comprising a multivalent aptamer having binding specificity to multiple targets.

11. An aptamer having binding specificity to PDGF, TGFβ1, and TGFβ2 useful as an anti-scarring agent.

12. A method of generating a multivalent aptamer comprising the step of, joining a plurality of component aptamers having been selected by SELEX process, wherein joining said aptamers preserves structural motifs required for binding activity of said component.

13. The method of Claim 12 wherein both component aptamer selections by the SELEX process is *de novo*.

14. The method of Claim 12 wherein component aptamer selection by the SELEX process is *de novo* for one of the plurality of component aptamers.

15. The method of Claim 12 where component aptamers are joined by chemical linking of the plurality of component aptamers.

16. The method of Claim 15 wherein the chemical linking is done with polyethylene glycol (PEG) as a linker.

17. The method of Claim 15 wherein the chemical linking is performed by a rationally designed oligonucleotide linker.

18. The method of Claim 15 wherein the chemical linking is performed by an oligonucleotide linker that is selected from a randomized region to minimize deleterious interactions with the component aptamer binding to a target.

19. The method of Claim 12 wherein the plurality of component aptamers are selected whereby binding of a first component aptamers activates binding of successive aptamer components.

20. The method of Claim 19 wherein said first component aptamer is selected to activate binding of a second aptamer component in a bivalent aptamer.

21. A multivalent aptamer capable of binding to more than one target.

22. The aptamer of claim 22 wherein the targets are on the same molecule.

23. The aptamer of claim 22 wherein the targets are on different molecules.

24. The aptamer of claim 23 wherein the targets are both involved in the same disease or disorder.

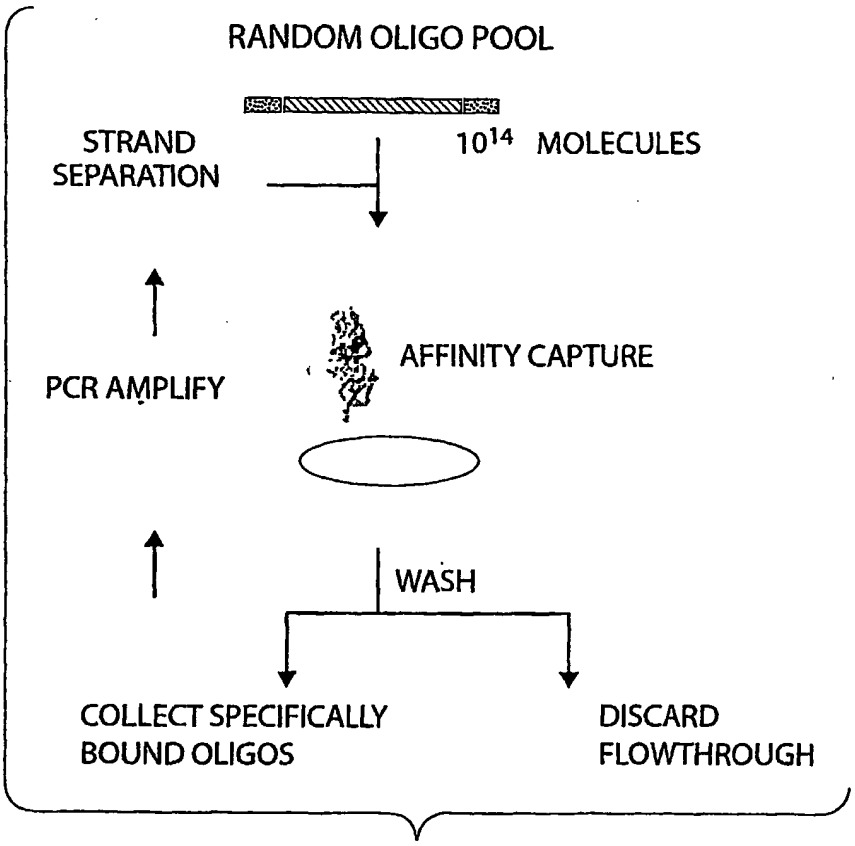

RANDOM OLIGO POOL
STRAND SEPARATION
10^{14} MOLECULES
PCR AMPLIFY
AFFINITY CAPTURE
WASH
COLLECT SPECIFICALLY BOUND OLIGOS
DISCARD FLOWTHROUGH

Fig. 1

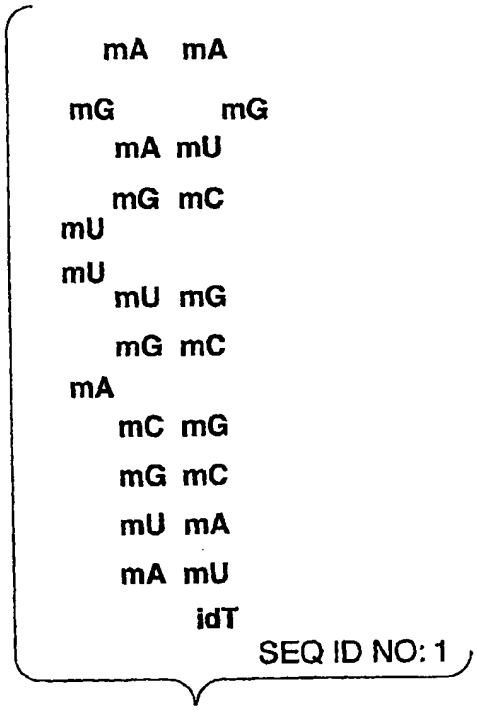

mA mA
mG mG
mA mU
mG mC
mU
mU
mU mG
mG mC
mA
mC mG
mG mC
mU mA
mA mU
idT
SEQ ID NO: 1

Fig. 2A

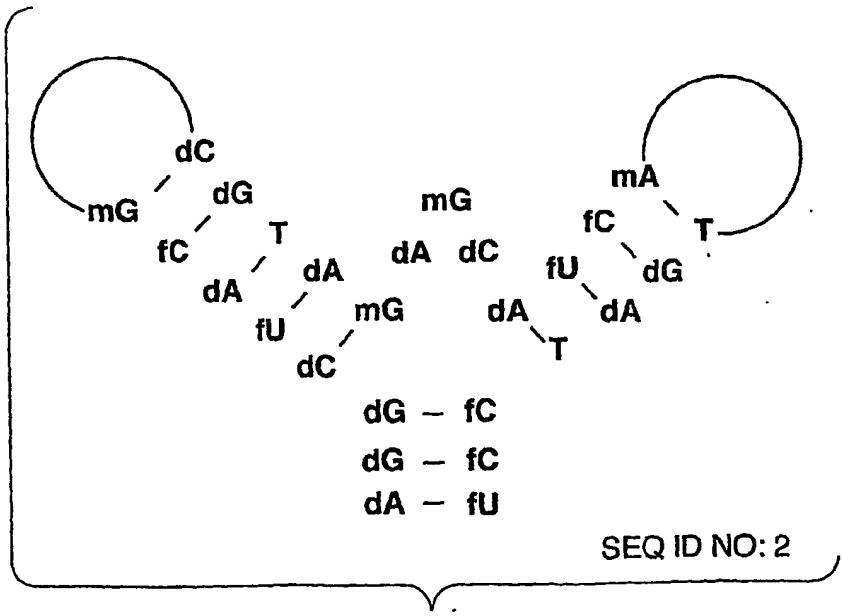
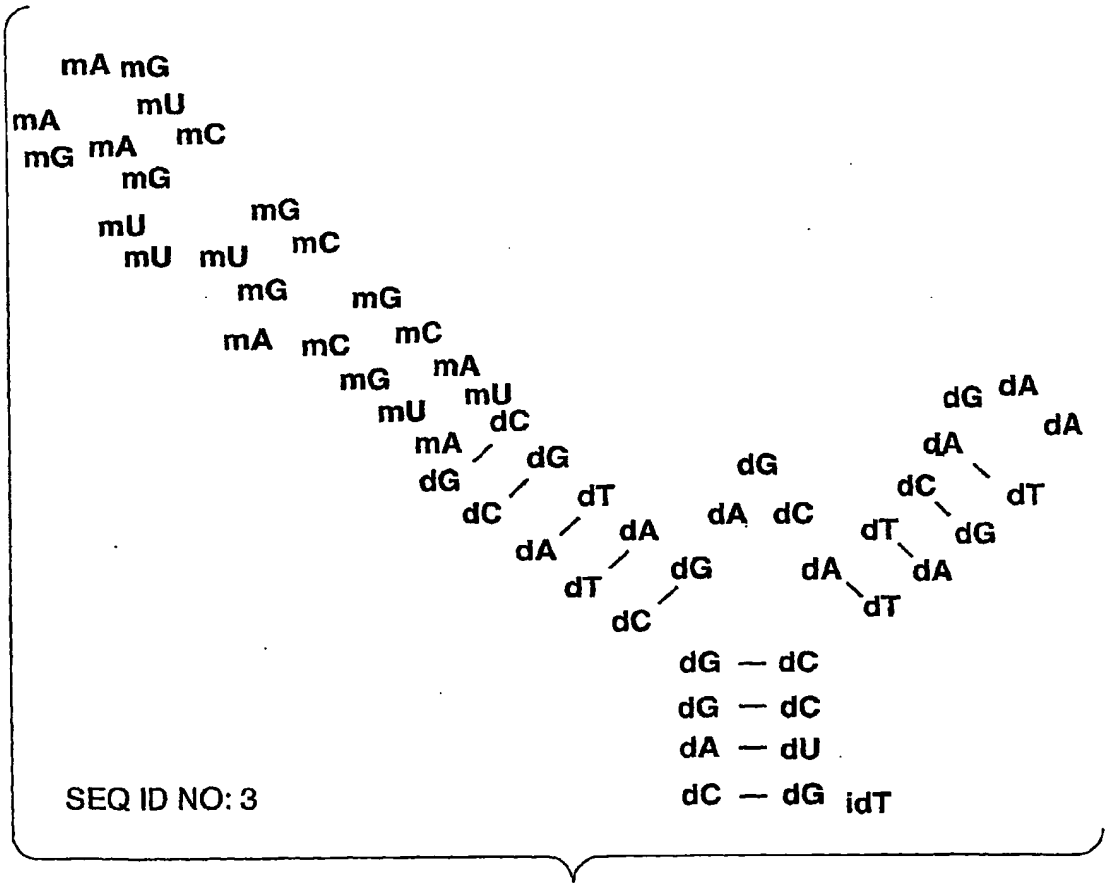

dC
mG
dG
fC
T
dA
dA
fU
mG
dC
mG
dA
dC
mA
fC
T
fU
dG
dA
dA
T
dG – fC
dG – fC
dA – fU
SEQ ID NO: 2

Fig. 2B


mA mG
mU
mA
mC
mG mA
mG
mG
mU
mC
mU mU
mG
mG
mA mC
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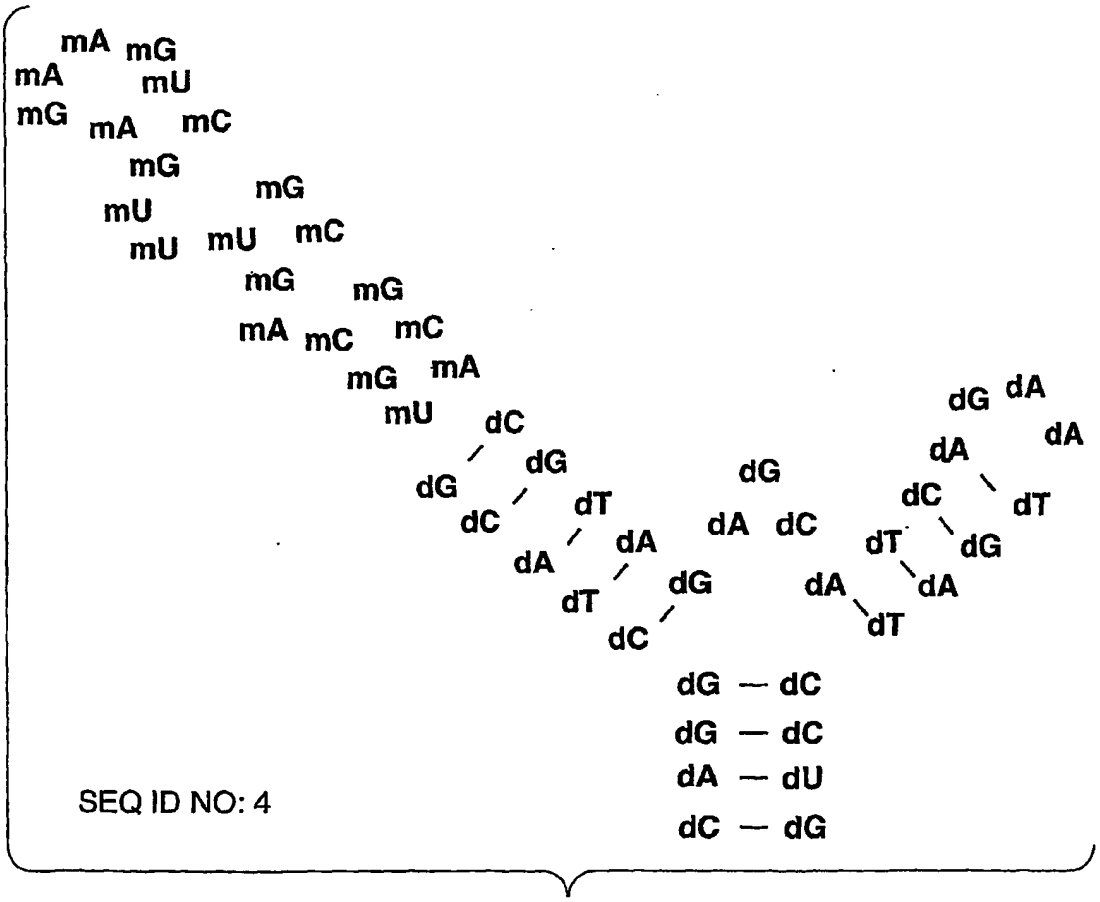
mA
mG
mU
mU
dC
mA
dG
dG
dT
dC
dA
dA
dT
dG
dC
dG
dA dC
dA
dT
dG
dA
dA
dT
dG dA
dA
dA
dC
dT
dT
dG
dG — dC
dG — dC
dA — dU
dC — dG idT
SEQ ID NO: 3

Fig. 2C


mA mG
mA mU
mG mA mC
mG
mG
mU
mU mU mC
mG mG
mA mC mC
mG mA
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dG dG
dG dC dT
dA dA
dT dG
dC
dG dA dC
dG dA dA
dA dC dT
dT dG
dA dA
dT
dG — dC
dG — dC
dA — dU
dC — dG
SEQ ID NO: 4

Fig. 3A

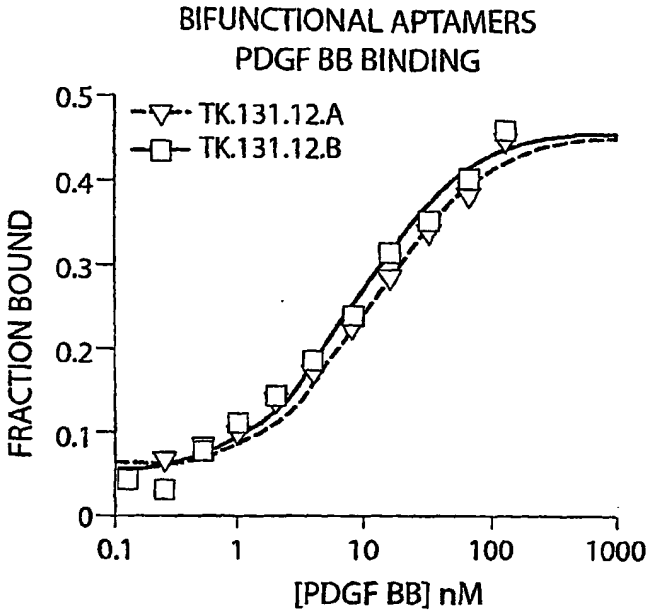

BIFUNCTIONAL APTAMERS
PDGF BB BINDING
TK.131.12.A
TK.131.12.B
FRACTION BOUND
0.5
0.4
0.3
0.2
0.1
0
0.1
1
10
100
1000
[PDGF BB] nM

Fig. 3B

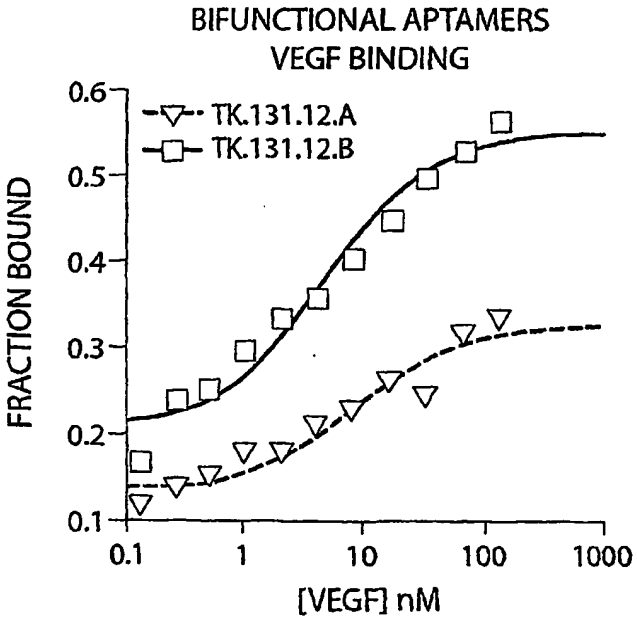

BIFUNCTIONAL APTAMERS
VEGF BINDING
TK.131.12.A
TK.131.12.B
FRACTION BOUND
0.6
0.5
0.4
0.3
0.2
0.1
0.1
1
10
100
1000
[VEGF] nM

Fig. 3C

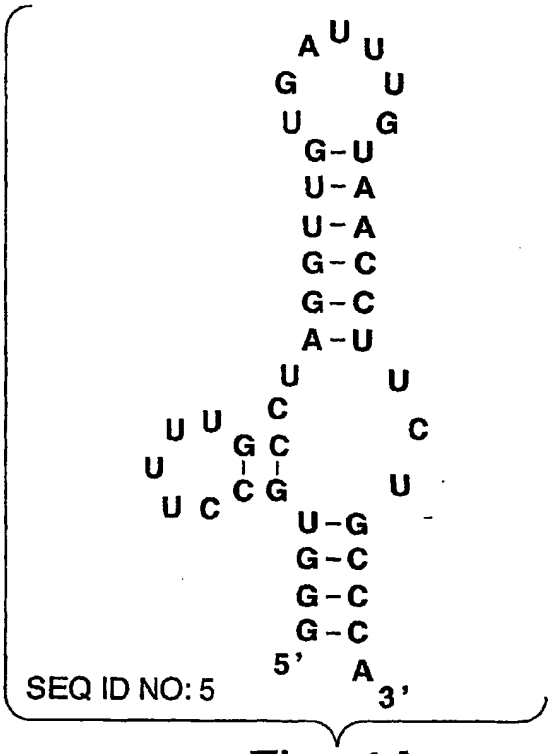


Fig. 4A

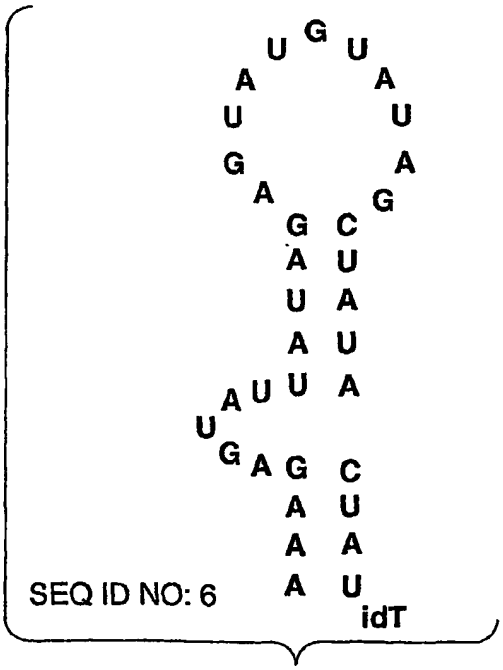
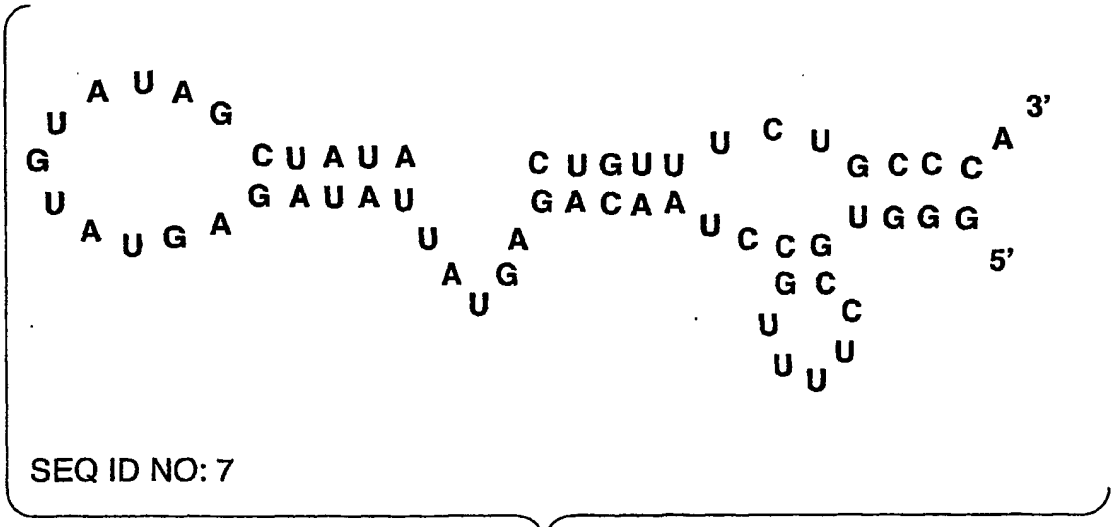


Fig. 4B



SEQ ID NO: 7

Fig. 4C

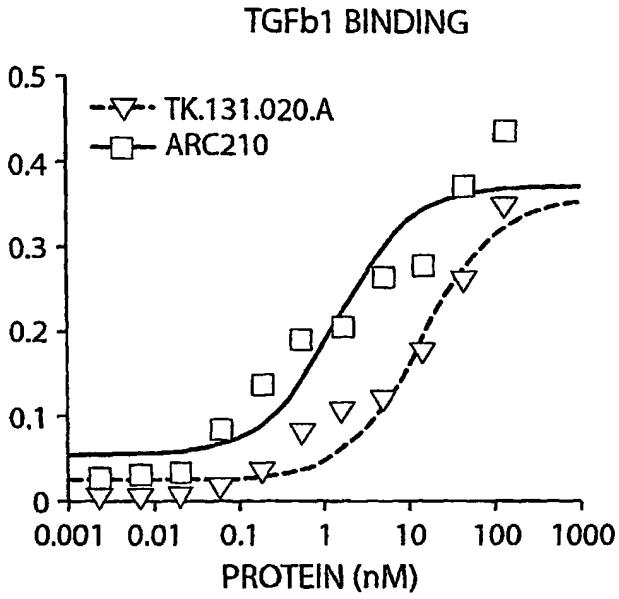

TGFb1 BINDING
TK.131.020.A
ARC210
0.5
0.4
0.3
0.2
0.1
0
0.001
0.01
0.1
1
10
100
1000
PROTEIN (nM)

Fig. 5A

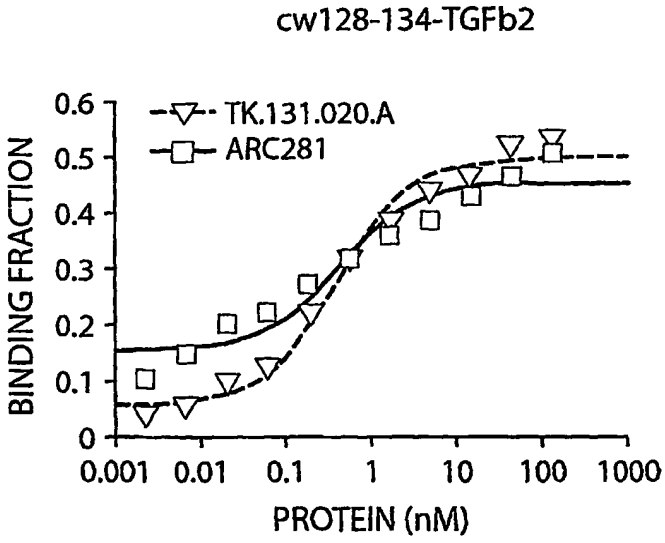

cw128-134-TGFb2
TK.131.020.A
ARC281
BINDING FRACTION
0.6
0.5
0.4
0.3
0.2
0.1
0
0.001
0.01
0.1
1
10
100
1000
PROTEIN (nM)

Fig. 5B

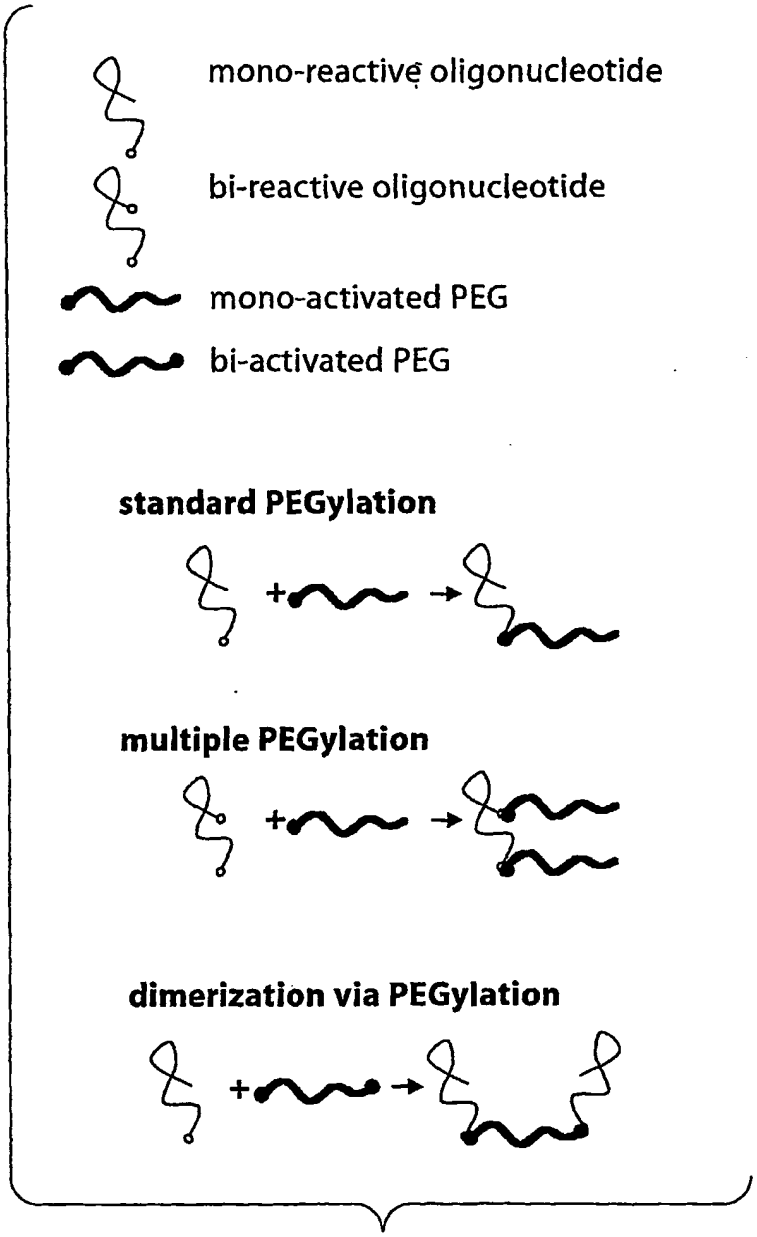

mono-reactive oligonucleotide
bi-reactive oligonucleotide
mono-activated PEG
bi-activated PEG
standard PEGylation
multiple PEGylation
dimerization via PEGylation

Fig. 6

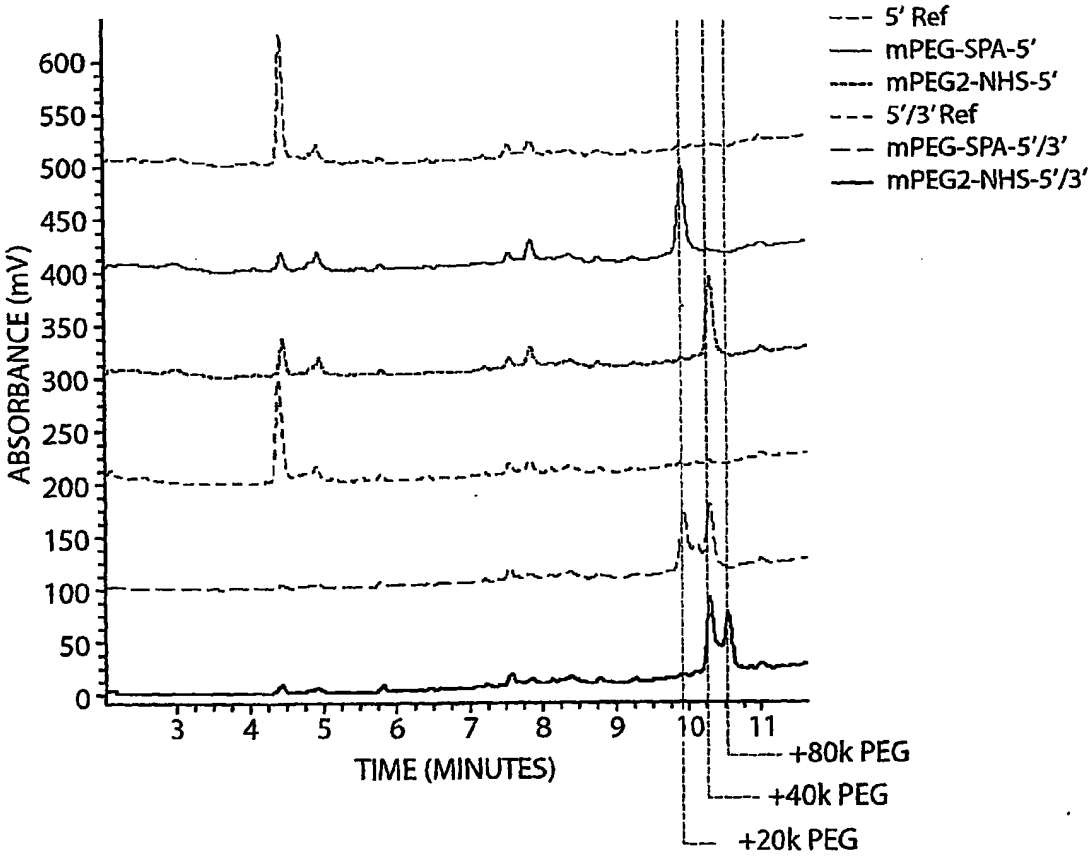
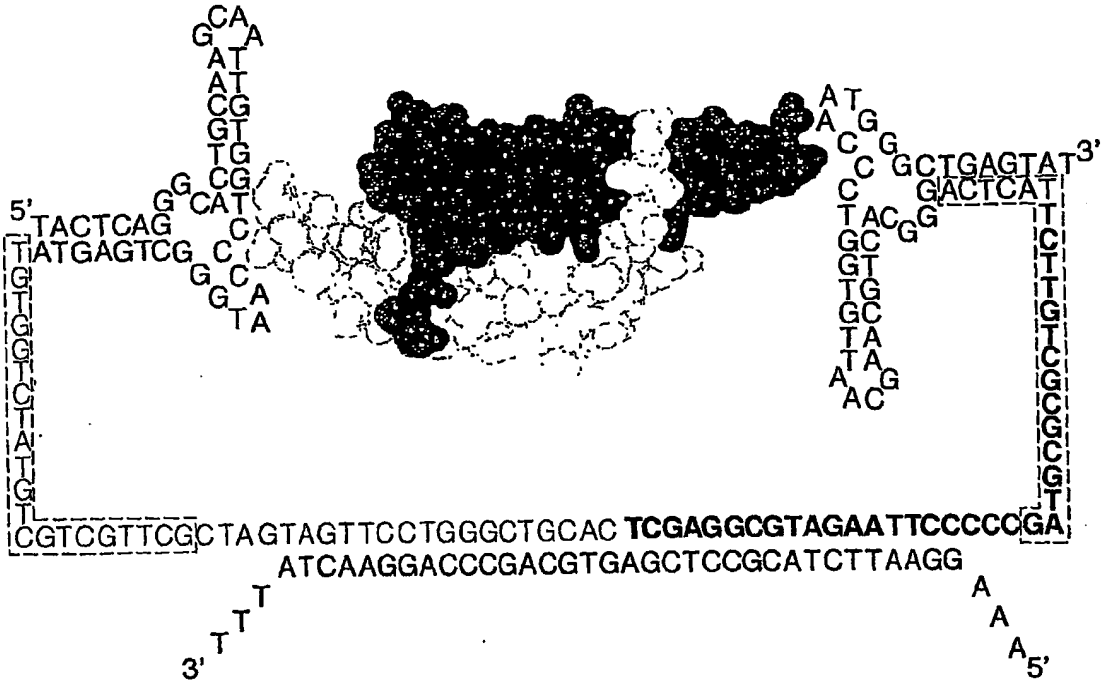

5' Ref
mPEG-SPA-5'
mPEG2-NHS-5'
5'/3' Ref
mPEG-SPA-5'/3'
mPEG2-NHS-5'/3'
ABSORBANCE (mV)
600
550
500
450
400
350
300
250
200
150
100
50
0
3
4
5
6
7
8
9
10
11
TIME (MINUTES)
+80k PEG
+40k PEG
+20k PEG

Fig. 7



SEQ ID NO: 8

Fig. 8A

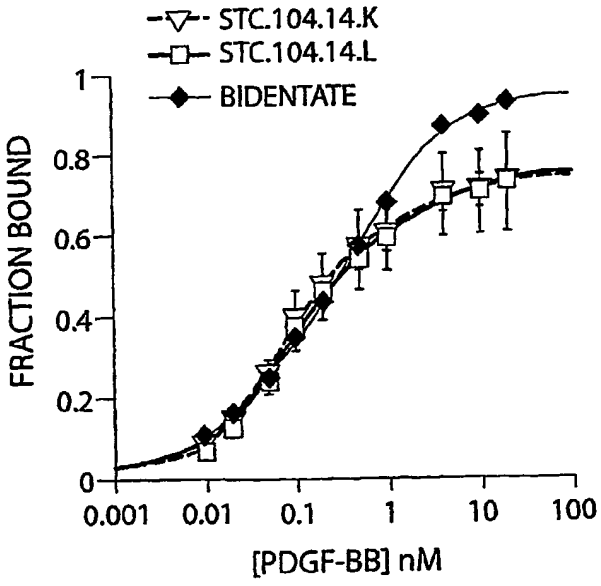
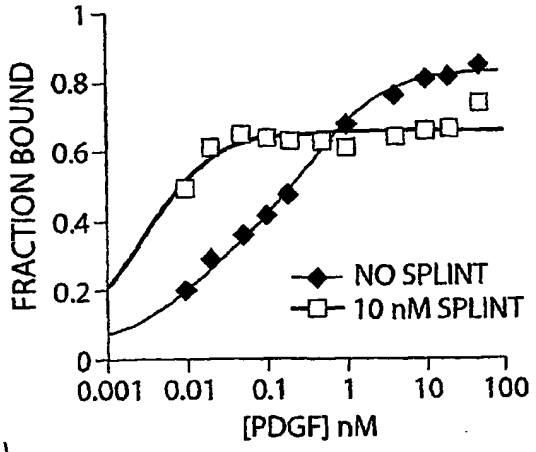
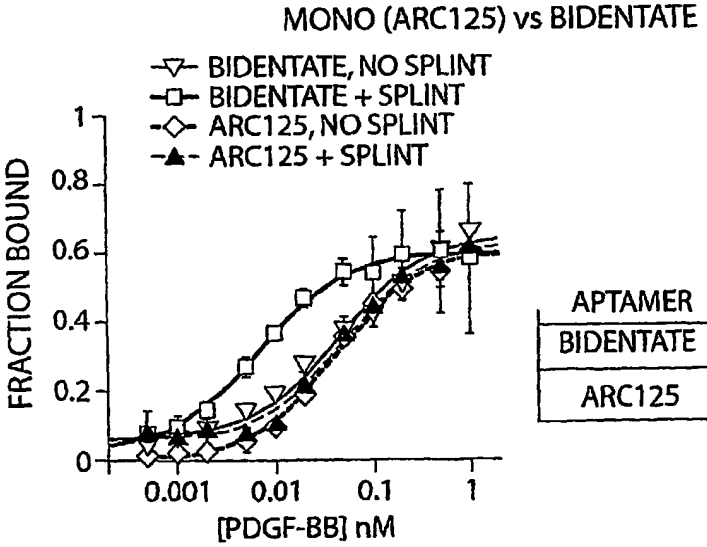


Fig. 8B



APTAMER	K_{D1} (pM)	K_{D2} (pM)
NO SPLINT	11 (30%)	460 (45%)
10 nM SPLINT	<10 (60%)	N/A

Fig. 9A



APTAMER	K_D (pM) NO SPLINT	K_D (pM) + SPLINT
BIDENTATE	47 ± 9	7 ± 2
ARC125	40 ± 1	49 ± 8

Fig. 9B

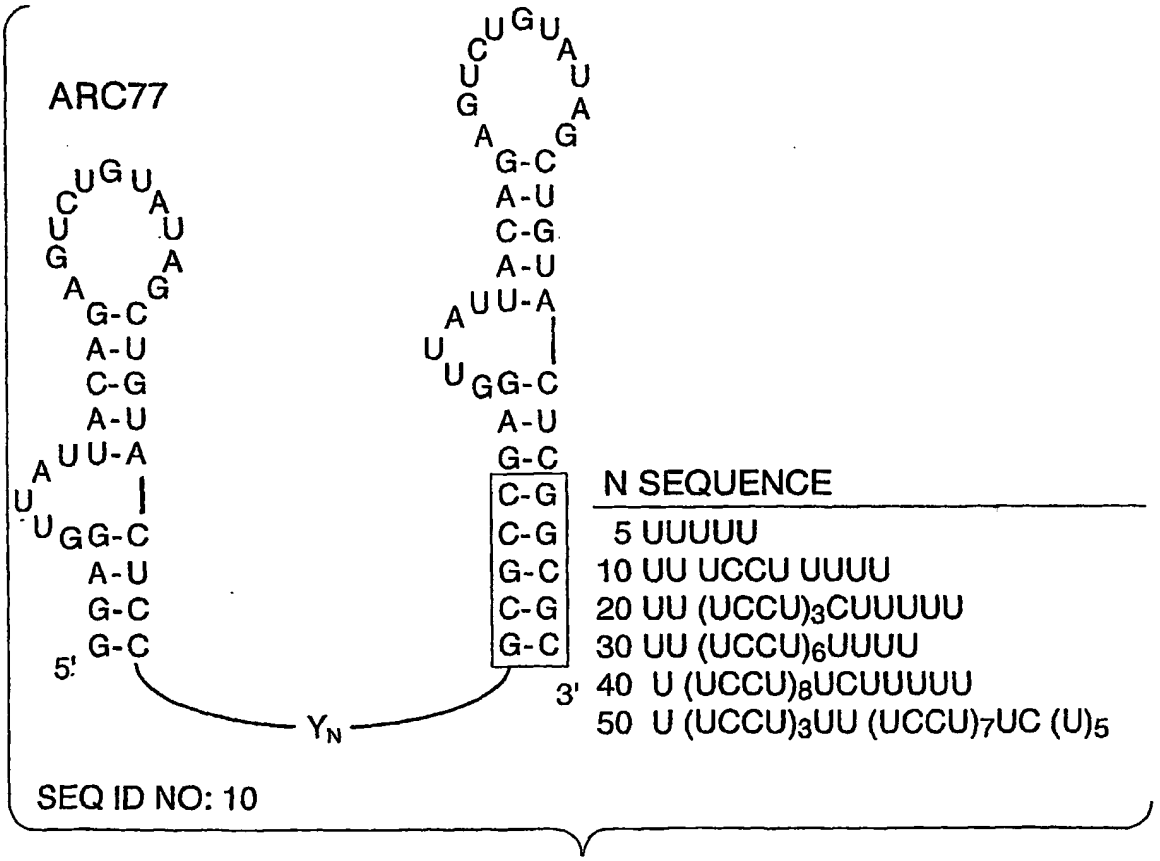
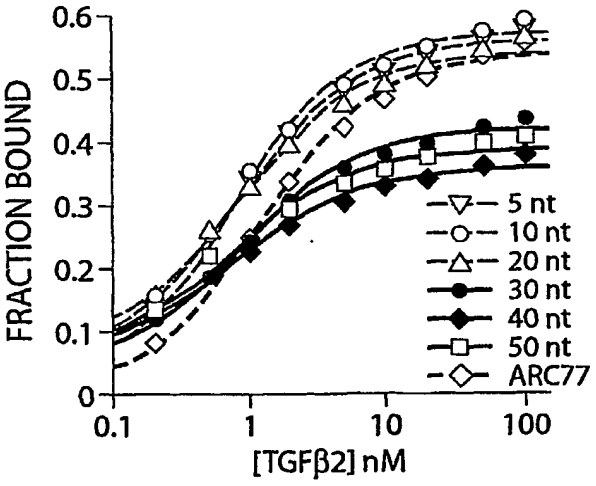

ARC77
5'
3'
Y_N
N SEQUENCE
5 UUUUU
10 UU UCCU UUUU
20 UU $(UCCU)_3CUUUUU$
30 UU $(UCCU)_6UUUU$
40 U $(UCCU)_8UCUUUUU$
50 U $(UCCU)_3UU\ (UCCU)_7UC\ (U)_5$
SEQ ID NO: 10

Fig. 10



CONSTRUCT	K_D (nM)
5 nt	0.8
10 nt	0.8
20 nt	0.8
30 nt	0.9
40 nt	0.8
50 nt	0.7
ARC77	1.2

Fig. 11